Universidade de Lisboa

Faculdade de Farmácia

Development of a topical mucoadhesive ocular delivery system for ceftazidime

Raquel Sofia D'Abreu Calado

Dissertação orientada pela Professora Doutora Lídia Maria Diogo Gonçalves e, coorientada pela Professora Doutora Ana Francisca Simão Bettencourt

Mestrado Ciências Biofarmacêuticas

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“The best way to predict your future is to create it”

Abraham Lincoln

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Agradecimentos

Após a finalização dos estudos conducentes ao grau de mestre, um dos grandes desafios que me propus ultrapassar, é importante fazer agradecimentos a todos os que tornaram possível a conclusão desta etapa.

Em primeiro lugar um especial agradecimento, a quem desde inicio aceitou ser minha Orientadora, Professora Doutora Lídia Maria Diogo Gonçalves, que me acompanhou sempre de forma incansável e colocando à minha disposição todos as condições necessárias ao desenvolvimento do projeto. A sua boa disposição, capacidade de motivação e conhecimentos científicos tornaram esta experiência muito gratificante.

À Professora Doutora Ana Francisca Simão Bettencourt, que aceitou ser minha Co-Orientadora, sempre pronta a ajudar, com sugestões pertinentes e sempre pronta a incentivar em momentos de maior desânimo.

Ao Professor Doutor António José Leitão das Neves Almeida. Líder do grupo Nano BB, que me permitiu conhecer e trabalhar no "Lab 112".

À minha família e amigos que foram o suporte ao longo desta caminhada, mesmo quando não estive tão presente, me mantiveram focada no objetivo final. Sem a vossa ajuda não teria sido possível.

Abstract

Bacterial keratitis is an infectious disease of the cornea that is characterised by inflammation. The common pathogens associated with this disease include *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Pseudomonas aeruginosa, Streptococcus pneumonia* and *Serratia* species, causing 90% of the bacterial keratitis cases. Currently, treatment of bacterial infections and inflammation in the eye has the problem of anatomic barriers and the delicate nature of the eye. Local drug applied to the eye represents a non-invasive, safe and less painful solution than surgery, laser treatments or eye injections.

Ceftazidime is a third generation cephalosporin antibiotic which is effective mainly against pathogens that are usually responsible for ophthalmological infections. Ceftazidime offers a good coverage against *Pseudomonas aeruginosa* as well as resistance to several types of beta lactamases.

The aim of the present work was to prepare a mucoadhesive nanoparticle eye drop formulation containing ceftazidime to treat eye infections.

The first step for the development of the eye drop formulation was to prepare a vehicle for nanoparticles. Two different polymers were selected: carboxymethyl cellulose and hydroxypropylmethyl cellulose, both in low and high viscosity. The selection was based in studies of viscosity, zeta potential and interaction with the nanoparticles. After the tests, the work continued with hydroxypropylmethyl cellulose.

The development of mucoadhesive chitosan-nanoparticles are proposed as effective delivery systems for ceftazidime through ocular epithelium, taking advantage of the favourable biological properties of hyaluronic acid and chitosan to prolong precorneal residence time of the antibiotic, enhancing drug accumulation and permeation. Nanoparticles were prepared by ionotropic gelation between sodium tripolyphosphate and chitosan with the objective to encapsulate ceftazidime.

The formulations were characterized in terms of pH, osmolality, viscosity, zeta potential, particle size distribution and encapsulation efficiency. In addition, the *in vitro* release and permeation studies were performed and the results suggest a prolonged drug release from the nanoparticles. The results of nanoparticles interaction with mucin show their mucoadhesivity and ability to interact with the ocular surface increasing the drug

residence time in the eye. The prepared nanoparticles were subjected to stability and microbiological studies with satisfactory results.

In conclusion, Chitosan/TPP-Hyaluronic Acid nanoparticles proved to be a promising platform for ceftazidime delivery in the eye.

Keywords: eye, ophthalmic drug delivery, chitosan nanoparticles, bacterial keratitis, ceftazidime.

Resumo

O olho é um órgão do corpo humano com uma estrutura e anatomia complexas por apresentar diversas barreiras que impedem que os fármacos possam penetrar para o tratamento das diversas afeções oculares. As barreiras estão presentes tanto ao nível do segmento anterior como posterior. Geralmente, os fármacos são administrados recorrendo à administração tópica e às formas farmacêuticas comuns mas as características únicas dos tecidos oculares e os mecanismos de defesa do globo ocular dificultam a administração de fármacos a este nível, obtendo-se uma baixa resposta terapêutica. Um dos desafios das novas formulações terapêuticas de administração ocular é aumentar a biodisponibilidade dos fármacos administrados topicamente assim como a sua eficácia terapêutica. A administração tópica ocular é desde sempre um desafio devido à dificuldade em manter concentrações adequadas de fármaco no local de aplicação durante tempo suficiente de modo a obter um adequado efeito farmacológico, o que requer repetidas aplicações do fármaco.

Estes objetivos podem ser atingidos através do recurso a estratégias que aumentem o tempo de residência pré-corneal, a mucoadesividade e a penetração através dos tecidos.

A queratite bacteriana é uma afeção ocular que afeta milhões de pessoas em todo o mundo causando problemas oculares graves podendo mesmo causar cegueira. Esta infeção conduz à destruição da córnea e tem como principais agentes patogénicos *Staphylococcus aureus*, *Staphylococcus* coagulase-negativo*, Pseudomonas aeruginosa, Streptococcus pneumonia* e *Serratia.*

O projeto desenvolvido visa utilizar a ceftazidima, antibiótico da classe das cefalosporinas de terceira geração, que atua na inibição da síntese da parede bacteriana, no combate ao principal agente causador da queratite bacteriana, mas não da forma convencional em que são normalmente utilizados.

Para melhorar o tempo de residência do fármaco, foram desenvolvidas nanopartículas poliméricas. Estas nanopartículas foram desenvolvidas por gelificação inotrópica entre quitosano e tripolifosfato às quais foi adicionado ácido hialurónico. Os componentes foram selecionados com base nas suas características de biodegradabilidade, biocompatibilidade e mucoadesão com as glicoproteínas da córnea e conjuntiva. O ácido

hialurónico foi selecionado pela sua interação com os recetores CD44 presentes na córnea e conjuntiva oculares facilitando a penetração das nanoparticulas a nível ocular.

Para o desenvolvimento das nanopartículas várias proporções dos três principais componentes foram sendo testadas variando a sua quantidade, proporções e pH do meio. Estas variações foram necessárias para verificar as condições ideais para que, por um lado ocorresse a formação das nanopartículas e por outro que estas se mantivessem estáveis e não precipitassem.

Para que as nanopartículas possam ser corretamente utilizadas têm que ter um veículo que permita a sua administração. O veículo foi desenvolvido utilizando polímeros mucoadesivos derivados da celulose, a carboximetilcelulose e hidroxipropilmetilcelulose utilizados também como agentes viscosificantes e que foram testados em diferentes concentrações. Testes de viscosidade, potencial zeta e compatibilidade com as nanopartículas produzidas foram desenvolvidos para chegar ao polímero e concentração ideais para funcionar como veículo.

As formulações foram caracterizadas em termos de pH, osmolalidade, viscosidade, potencial zeta, distribuição de tamanho das nanoparticulas e eficiência de encapsulação do fármaco.

Adicionalmente foram realizados estudos *in vitro* de libertação e cedência que demonstram uma libertação continuada do fármaco a partir das nanopartículas formuladas. Estudos de viscosidade e potencial zeta foram também desenvolvidos na avaliação da interação entre a formulação e a mucina ocular comprovando-se a mucoadesividade que permite prolongar o tempo de residência ocular e a libertação do fármaco. As nanopartículas desenvolvidas foram sujeitas a estudos microbiológicos demonstrando ter atividade contra agentes causadores da queratite bacteriana. Os estudos de toxicidade desenvolvidos demonstraram resultados satisfatórios pois indicaram que a formulação não teve efeitos citotóxicos nas linhas celulares testadas.

Em conclusão, as nanopartículas de quitosano/TPP-Ácido Hialurónico são uma promissora formulação ocular para a veiculação da ceftazidima, promissora no tratamento da queratite bacteriana.

Palavras-chave: olho, administração ocular de fármacos, nanopartículas de quitosano, queratite bacteriana, ceftazidima.

Abbreviations

CFT	Ceftazidime
CMC	Carboxymethyl cellulose
CS	Chitosan
CS/TPP/HA NP	Chitosan-TPP-hyaluronic acid nanoparticles
D.L.	Drug loading
EDTA	Ethylenedinitrilotetraacetic acid disodium salt dihydrate
E.E.	Encapsulation efficiency
HA	Hyaluronic acid
HPMC	Hydroxypropylmethyl cellulose
Jss	Steady-state flux
Kp	Permeability coefficient
LMW	Low molecular weight
MIC	Minimum inhibitory concentration
NP	Nanoparticles
OD	Optical Density
PBS	Phosphate buffered saline
PVA	Polyvinyl alcohol
PDI	Polydispersity index
TEM	Transmission electron microscopy
TPP	Sodium tripolyphosphate
UV	Ultraviolet
WHO	World Health Organization
ZP	Zeta Potencial

1. Introduction

The human eye is a small organ that provides sense of sight, allowing the perception of shapes, colours and dimensions around the world. In spite of constant environmental changes, the eye has the ability to adapt to new conditions (Kansara *et al*, 2007). It is composed of several different structures and layers, with specific physiological roles. One of the most important functions of these structures is to protect the ocular globe against external aggression. For this protection, there are tight cellular barriers in the anterior and posterior parts of the eye that restrict the uptake of fluids and prevent penetration of foreign bodies (Almeida *et al*, 2014).

According to the World Health Organization, a wide diversity of eye diseases affects millions of people around the world and has devastating effects on individuals, leading to visual injury and possible ocular blindness that instigate a decline in quality of life (WHO).

Because of all the ocular barriers the design of targeted ocular drug delivery systems to overcome them remains one of the greatest challenges in pharmaceutical sciences. Static barriers (different layers of cornea, sclera, and retina including blood aqueous and blood–retinal barriers), dynamic barriers (choroidal and conjunctival blood flow, lymphatic clearance, and tear dilution), and efflux pumps in conjunction pose a significant challenge for delivery of a drug alone or in a dosage form, especially to the posterior segment (Gaudana *et al*, 2010).

Topical instillation is the most preferred non-invasive route of drug administration to treat diseases affecting the anterior segment. Conventional dosage forms such as eye drops account for 90% of the marketed ophthalmic formulations. The reason may be attributed to ease of administration and patient compliance (Bravo-Osuna *et al*, 2016). Nonetheless, the ocular bioavailability is very low with topical drop administration. Numerous anatomical and physiological constraints such as lacrimation and blinking tear dynamics, drainage by gravity, nasolacrimal drainage (the residence volume of the tear film is 7–10 μL, while the human cul-de-sac may contain about 30 μL volume). The excess is removed through the nasolacrimal drainage system (Patel *et al*, 2015). Less than 5% of topically applied dose reaches to deeper ocular tissues. It is difficult to achieve therapeutic drug concentration into posterior segment ocular tissues following topical eye drops instillation because of the above mentioned barriers. The drug can be delivered to the posterior

segment ocular tissues by different modes of administrations such as intravitreal injections, periocular injections, and systemic administration.

One way to overcome some of the eye's natural anatomical barriers is to take advantage of the ocular surface mucosal layer and use its structure to aid in biopharmaceutical adherence and penetration by incorporating mucoadhesive substances into the delivery system. These mucoadhesive substances can be integrated into a range of different delivery systems and used in conjunction with a variety of biopharmaceuticals to make an effective device for ocular pharmaceutical delivery. Among different drug delivery systems, nanoparticles are being intensively systems. So, nanoparticles are a system that have been designed to overcome the barriers, increasing the drug penetration at the target site and prolong the drug levels by few intervals of drug administrations in lower doses without any toxicity compared to the conventional eye drops (Sheardown and Lorentz, 2014).

1.1-Anatomy of the Eye

A close examination of the physiology and anatomy of the eye (Figure 1) is of great importance to understand the challenges associated with ocular drug delivery.

The eyeball is enveloped by a three layer covering which wraps the internal structures.

The innermost layer is the retina, middle is the uveal coat, and the outermost layer is the sclera. The sclera is composed of tough fibrous tissue, which covers the posterior section of the eyeball and continues into the anterior eye to form the clear transparent cornea (Sheardown and Lorentz, 2014).

The structure of the eye can be divided in two segments, namely anterior and posterior segments (Kansara *et al*, 2007).

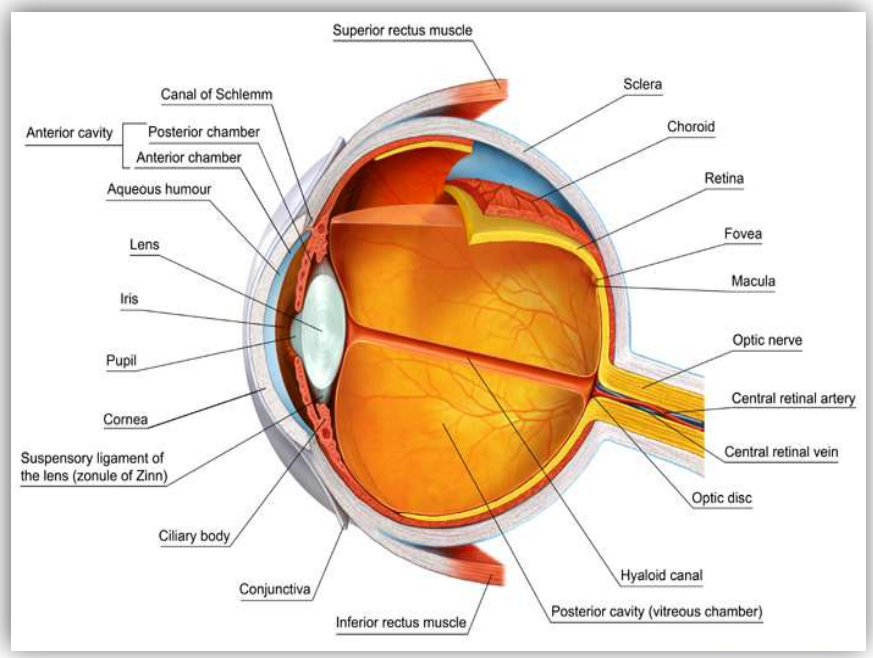


Figure 1.1 - Eye anatomy (Reference:/www.virtualmedicalcentre.com/anatomy/the-eye-andvision/ 28)

The anterior segment comprises two chambers, i.e the anterior (between the cornea and iris) and posterior (between iris and lens). The anterior segment of the eye includes structures such as the lens, lachrymal system, iris, aqueous humor, ciliary body, pupil, conjunctiva, and the cornea (Sheardown and Lorentz, 2014).

The conjunctiva is a thin transparent membrane which covers the inside surface of the eyelids and extends onto the anterior surface of the eye to cover the sclera, meeting the corneal epithelium at the limbus. The conjunctiva is vascular and it is composed of three layers: epithelium, substantia propria, and the submucosa and it is divided into two main regions: the palpebral and bulbar conjunctiva (Patel *et al*, 2015). The surface epithelial cells of the conjunctiva are connected by tight junctions and have scattered goblet cells that secrete approximately 2-3 µL of mucous per day, which covers the eye surface, and has the function of hydration, cleaning and lubrication and serves as a defence against pathogens (Almeida *et al*, 2014).

The cornea is a major barrier for traditional topical drug delivery in the treatment of anterior segment diseases. It is a transparent, avascular and highly innervated tissue, with

11.7 mm diameter and a thickness of 0.5-0.7 mm; the cornea is thicker in the centre than in the limbus (Mun *et al*, 2014).

The five layers of the cornea are the epithelium, Bowman's layer, stroma, Descemet's membrane, and the endothelium. The epithelium is the anterior-most layer of the cornea consisting of 5–6 layers of cells, joined together by the presence of tight junctions and is able to regenerate following an injury The Bowman's layer is a cellular layer of the stroma that is not able to regenerate. The stroma is the thickest layer of the cornea composed of collagen fibres and 90% water which provide the cornea with its transparent properties. The Descemet's membrane is an elastic membrane which covers the endothelium. (Sheardown and Lorentz, 2014). The corneal epithelium layer possesses tight junction cells which limits the entry to small hydrophilic drug molecules following topical administration. Moreover, hydrophilic structure of stroma offers limited entry of lipophilic compounds. Presence of efflux proteins (P-glycoprotein and multidrug resistance protein) on the corneal epithelium also restricts the entry of xenobiotics to the anterior segment tissues. Pre-corneal drainage, tears washout and limited contact time are major challenges to the anterior segment drug delivery following topical administration. To be clinically effective topical formulation has to possess a balance between the hydrophilicity and lipophilicity with high contact time (Kansara *et al*, 2007).).

The iris is the coloured part of the eye which is located between the cornea and the lens and controls the size of the pupil. It has two main layers: the connective tissue rich stroma and the pigmented epithelium (Stjernschantz and Astin, 1993).

The crystalline located posterior to the iris, is attached to the ciliary body which contains the ciliary muscle that enables it to change its shape to allow for light to be focused on the retina (Sheardown and Lorentz, 2014)

Separates the aqueous and vitreous humor and is composed of three main parts: lens fibers, lens epithelium, and the lens capsule (Sheardown and Lorentz, 2014)

The aqueous humor is composed of a clear jelly-like fluid that fills the anterior segment of the eye, controls intraocular pressure, removes waste and provides nutrients to the surrounding tissues (Stjernschantz and Astin, 1993).

The crystalline is responsible for three main functions: secretion, distribution, and collection of tears. The lacrimal gland secretes tears due to the basic need to maintain the tear film, from reflex tearing due to stimulation such as irritation or temperature, or due to emotional tearing. In healthy individuals, basal tear production ranges from 0.5–2.2

μL/min, but this can increase to 300 μL/min for reflex tearing (Sheardown and Lorentz, 2014).

The posterior segment of the eye includes the retina, choroid, sclera, macula, fovea, optic nerve, and the vitreous humor.

The vitreous humor is a dense gelatinous substance that fills in the space between the posterior side of the lens and the retina. The production of vitreous humor is minor than the production of aqueous humor, and has a slow turnover. It main function it's the maintenance of the ocular pressure keeping the retina and crystalline located in the right places, being responsible for light refraction (Kumar *et al,* 2011).

The retina is a thin membrane which is composed of two layers: the outer pigmented epithelium and the inner neuro-epithelium. Overall, the retina is responsible for detecting light focused on the retina and converting it to nerve impulses which are sent through the optic nerve and into the brain. The retina is dense with photosensitive cells called cones and rods (Kumar *et al,* 2011).

In the intact retina occurs the absorption of drugs to the blood vessels or its transportation by the pigmented epithelium. In the case that the drugs are transported by the pigmented epithelium it can be absorbed by the vessels of the choroid (Kumar *et al,* 2011).

The choroid located posterior to the retina and the uvea and is responsible for delivering oxygen and nourishment to the retina. It is composed of four layers, is heavily vascularized, pigmented, and contains connective tissue (Sheardown and Lorentz, 2014).

The sclera's function is mainly protective and covers the bulk of the posterior part of the eyeball in a thick dense fibrous tissue and muco-polysaccharides. Not only does this protect the internal sensitive structures but it also provides a site for attachment for the ocular muscles and maintains the shape of the eyeball (Sheardown and Lorentz, 2014).

The optic nerve is the second cranial nerve and is responsible for vision. Each nerve contains approximately one million fibres transmitting information from the rod and cone cells of the retina (Kumar *et al,* 2011).

1.2- Structure of the Tear Film

The tear film is a complex multilayered film that covers the anterior surface of the conjunctiva and cornea. It is thought to provide several unique roles and therefore its composition needs to be tightly regulated. Its thickness is approximates to be 3 μm.

(Sheardown and Lorentz, 2014). It is composed by three layers with different origins and composition (lipid, aqueous, and mucous layers) (Figure 2). The lipid layer is produced by the meibominan glands and it's composed by sterols, fatty acids, glycerides and esters, polar lipids, the aqueous layer becomes from the lacrimal glands and the main composition are proteins, lactoferrin, salts, glucose, urea and water. The mucin layer has origin in the conjunctival goblet cells, glands of Moll and Krasse.

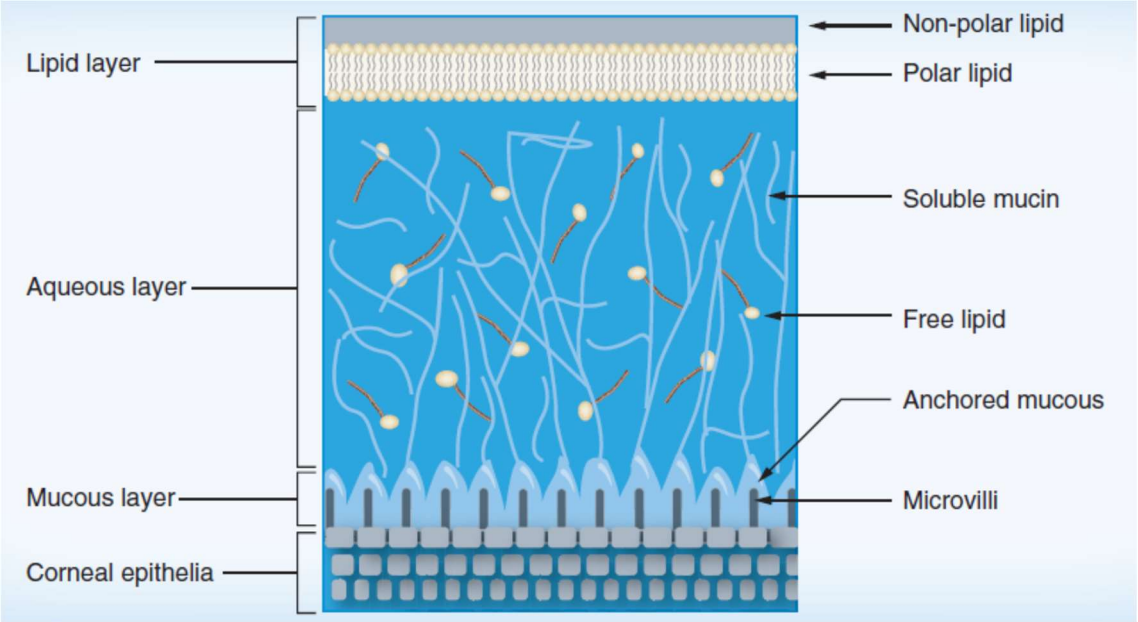


Figure 1.2 - Tear film composition (Reference: Morrison and Khutoryanskiy, 2014)

Table 1.1 - Tear film layers and functions (Morrison and Khutoryanskiy, 2014)

Tear film layer	Function
Lipid layer	To prevent evaporation and to provide a barrier To provide a smooth optical surface for the refraction light To act as a lubricant to aid the eyelid movement To form a barrier against tear film contamination To provide a surfactant layer between the non-polar lipid layer and the aqueous layer
Aqueous layer	To prevent tear overflow To create a favorable environment for the corneal epithelial cells, carry oxygen and nutrients to and from the cornea, and allow cell movement over the ocular surface To wash away toxic substances and debris during blinking To aid in antimicrobial activity through the tear film proteins (lipocalin, lactoferrin, lysozyme, and IgA) Growth factors present in this tear film phase play a significant role in corneal physiology
Mucous Layer	To act as a pathogen barrier using the ocular surface glycocalyx Mucin is a lubricant, which allows the eyelid and conjunctiva to move smoothly over each another during blinking and ocular movements Mucus threads protect the conjunctiva and cornea from injury by coating foreign bodies with a slippery mucus Mucus aids in glycocalyx formation and wetting the ocular surface Mucus helps overcome the hydrophobicity of the corneal surface

1.3- Ocular mucin

Since the concept of mucosal drug delivery involves an intimate relationship between the mucin and the delivery system, it is important to understand the composition of the mucous layer. Mucins are large glycoproteins which are mainly composed of a protein core, carbohydrates and are well glycosylated. There are two main types of mucin:

secreted mucins and membrane-associated mucins. Secreted mucins can be further divided into soluble and gel-forming mucins.

Ocular membrane-associated mucins, such as MUC1 and MUC16, are structured to have short cytoplasmic tails, a heavily glycosylated extracellular domain which can reach the glycocalyx, and a hydrophobic domain which spans the membrane and anchors the mucin. These membrane-associated mucins help create a hydrophilic barrier and may have their own signalling abilities. Ocular secreted gel-forming mucins, such as MUC 2, 5AC, and 5B, are the largest glycoproteins, contribute to the viscoelastic properties of mucus, and help trap particles and bacteria. Ocular secreted soluble mucins, such as MUC 7, are the smallest mucins found in the tear film (Sheardown and Lorentz, 2014).

Approximately 2.5 μL of mucus is produced every day, and it is thought that the mucus layer is replenished at least once daily (Gan *et al*, 2013).

1.4- Routes of drug delivery to the eye

There are different possible routes of drug delivery into the ocular tissues, and the selection of the route of administration depends primarily on the target tissue (Urtti, 2006).

1.4.1- Topical administration

It is the most commonly used route of drug administration for the treatment of anterior segment complications. However, there are many barriers blocking successful and effective topical delivery of biopharmaceuticals to the eye. These challenges include ocular anatomical barriers due to the extraordinary and complex structure of the eye, biopharmaceutical barriers related to the biopharmaceutical's properties, and patient barriers related to comfort, compliance, and self-administration of the treatment (Sheardown and Lorentz, 2014).

Advantages of this route are relative simplicity of the formulations, minimal storage limitations and ease of drug instillation. Disadvantages include limited drug concentration for lipophilic agents, pre-corneal losses and the barrier function of the cornea. However, regardless of the ease of access to the eye for topical application of medication, efficient ocular drug delivery is hampered by a series of clearance mechanisms that protect the ocular structures from foreign matter. Upon administration of traditional eye drops they are immediately diluted in the tear film, followed by very quick elimination by the action of blinking, wash out by tears and nasolacrimal drainage. After instilling eye drops, there

remains a very short time where any residual medication is in contact with the cornea, during which time there is opportunity for the drug to penetrate into the eye; however, due to poor corneal permeability only a very small portion of active pharmaceutical ingredient will be capable of crossing the cornea. Typically, less than 5% of the applied drug penetrates the cornea and reaches intraocular tissues (Le Bourlais *et al*., 1998).

1.4.2- Systemic administration

For the systemic administration, the blood–aqueous barrier and blood–retinal barrier are the major barriers for anterior segment and posterior segment ocular drug delivery, respectively. For effective systemic deliver, such as oral or intravenous, a relatively high drug concentration needs to be circulating in the blood plasma in order to achieve a therapeutically effective dose in the eye, bioavailability is less than 2% and can require high dosage concentrations which can cause toxicity (Morrison and Khutoryanskiy, 2014).

1.4.3- Intravitreal administration

Intravitreal injection of drugs into the eye involves direct injection of the formulation, in the form of solution, particles, suspension, depot or implants, into the posterior segment (Kuppermann and Loewenstein, 2010). Direct drug administration into the vitreous offers distinct advantage of more straightforward access to the vitreous and retina, providing increased drug concentrations at the neural retina and minimizing systemic side effects (Urtti, 2006; Kuppermann and Loewenstein, 2010)

1.4.4- Periocular delivery

The periocular route includes subconjunctival, subtenon, retrobulbar and peribulbar administration and is comparatively less invasive than intravitreal route, being considered the least painful and the most efficient route of drug delivery to the posterior eye (Gaudana *et al*., 2010; Kuppermann and Loewenstein, 2010). Drug solutions are placed in close proximity to the sclera which results in high retinal and vitreal concentrations as the periocular route enables the deposition of molecules against the external surface of the sclera because it is made up of fibrous tissue, which offers less resistance to permeability of drugs (Gaudana *et al*., 2010; Kuppermann and Loewenstein, 2010).

1.5- Strategies to improve ophthalmic drug delivery

In a general way, for the treatment of ocular pathologies, there are used the conventional pharmaceutical forms presented as solutions, suspensions, ointments and gels (table 2). However, with the use of these ones only about 5% of the dose reaches the target. So, to get a therapeutic effect it is necessary to give repeated or high doses. This both situations can cause an increase of interactions or exacerbation of local and/or systemic side effects with potent drugs such as timolol (a beta-blocker) (Lallemand *et al* , 2013).

Table 1.2 - Advantages and disadvantages of conventional ocular drug delivery systems (Adapted from Patel et al, 2015)

	Advantages	Disadvantages
Solution	Easy and practical administration	Elimination by tears and nasolacrimal drainage Pre-corneal elimination Low bioavailability Low pharmacological action Repeated administrations
Suspension	Prolonged contact with ocular mucosa Useful for low solubility drugs	Irritation due to particle size
Gel	Less blurred vision when compared with ointments Incorporates a great variety of drugs	Adherence to the eyelid
Ointment	Long contact time with ocular mucosa No drainage by tears Better stability and bioavailability	Blurred vision Adherence to the eyelid

In view of the problems of conventional drug delivery systems, it was necessary to develop new pharmaceutical systems that allow the drug to get the tissue in effective concentrations. New pharmaceutical systems have to release the drug in an optimized way in the local of action. Consequently, there is a need for new formulations that will improve efficacy while also limiting the risk of local side effects (Mudgil *et al,* 2012)

1.6- Mucoadhesion and characterization of mucoadhesive properties

One of the main aims currently in topical ocular drug and biopharmaceutical delivery is to increase the residence time of the drug at the ocular surface, to increase drug uptake, diffusion and transport. This can be done by changing the characteristics of the delivery system, for example: by making a liquid system more viscous, but this creates its own set of problems for the patient, including blurred vision, which may jeopardize its success. However, if the delivery system could specifically bind to the ocular surface, this could increase biopharmaceutical residence and release time, decrease the concentration and volume required, and also decrease the frequency of treatment administration. This is the aim of ocular mucosal drug and biopharmaceutical delivery, by which the specific structural components of the mucin layer of the tear film are exploited to become an integral part in ocular delivery, which creates a more effective, efficient, and user friendly ocular delivery system (Sheardown and Lorentz, 2014).

An adhesive, is a material that attaches to another substrate surface and resists separation. Adhesion involves the formation of attractive bonds between two substrates that resist separation. Bioadhesion is a specific case of adhesion in which at least one of the two substrates involves a biological tissue. Furthermore, if the adherent substrate surface is a mucosal surface, e.g., a mucosal membrane, bioadhesion is specifically referred to as mucoadhesion (Wu *et al*, 2014).

Mucoadhesive formulations are capable of providing localized drug release in desirable regions like the eyes to enhance their clinical efficacy. The employment of mucoadhesive materials in formulations may modify the permeability of mucosal tissue or membranes and hence facilitate the adsorption of macromolecules (Singh and Rana, 2012).

Mucoadhesive controlled-release device can improve the effectiveness of a treatment by helping to maintain the drug concentration between the effective and toxic levels, inhibiting the dilution of the drug in the body fluids, and allowing targeting and localization of a drug at a specific site (Huang et al, 2000).

Mucoadhesion has shown renewed interest for prolonging the residence time of mucoadhesive dosage forms through various mucosal routes in drug delivery applications. Mucoadhesive-based topical and local systems have shown enhanced bioavailability. Mucoadhesive drug delivery gives rapid absorption and good bioavailability due to its considerable surface area and high blood flow (Shaikh *et al*, 2011).

Mucoadhesive drug delivery systems offer several advantages over other delivery systems including increased residence time of drug at the site of application, increased permeability of drug into the systemic circulation and enhanced bioavailability of the drug.

In general, the mucoadhesion phenomenon can be described in three steps: the first step involves wetting and swelling of the polymer to allow an intimate contact with the tissue, second, interpenetration and/or entanglement of the polymer and the mucin chains, and finally, the formation of weak chemical bonds leading to adhesion of the polymer to the mucosal surface. The interactions between a polymer and the mucous layer could be physical or mechanical bonds, secondary chemical bonds and covalent chemical bonds. Physical bonding implies the entanglement of mucin glycoproteins with the polymer chains, and the interpenetration of the mucin chains into the polymer matrix. Secondary chemical interactions include polar, van der Waals and hydrogen bonding. Covalent bonding could take place between the polymer and the mucosal substrate (Singh and Rana, 2012).

1.6.1- Mucoadhesion theories of polymer attachment

Mucoadhesion is a complex process and has not yet been fully understood and numerous theories have been developed to explain the phenomenon of mucoadhesion. No individual theory has been universally accepted as the singular mechanism by which bioadhesion occurs (Huang *et al,* 2000) though a combination of theories may be used to describe the phenomenon: electronic theory, wetting theory, adsorption theory, and diffusion theory.

1.6.1.1- Electronic theory

In the electronic transfer theory, mucoadhesion occurs as the result of the transfer of electrons between glycoprotein mucin network and the mucoadhesive polymer (Morrison and Khutoryanskiy, 2014). This electron transfer occurs because of the difference in structure between the bioadhesive and the glycoprotein chains in the mucus. Bioadhesion in this case is due to an attraction across the electrical double layer (Wu *et al*, 2014).

1.6.1.2- Wetting theory

The ability of mucoadhesive polymer to spread and develop intimate contact with the mucus layer is determinant for bond formation. It is best applied to liquid or low-viscosity

bioadhesives. It explains adhesion as an embedding process, whereby adhesive agents penetrate into surface irregularities of the substrate and ultimately harden, producing many adhesive anchors. Free movement of the adhesive on the surface of the substrate means that it must overcome any surface tension effects present at the interface (Shaikh *et al*, 2011). Adhesive forces between a liquid and solid enable a liquid drop to spread across the surface, whereas, cohesive forces within the liquid cause the drop to ball up and avoid contact with the surface. Generally, contact angles less than 90◦ indicate that the wetting of the surface is favourable, and the liquid tends to spread out to a large area. A contact angle greater than 90◦ indicates the wetting of the surface is unfavourable; the interaction among liquid molecules maintains the shape of the droplet and minimizes its contact area to the solid surface (Wu *et al*, 2014). The contact angle may be experimentally measured from which interfacial tension (γ) may be derived using the Young equation:

$$\gamma SG = \gamma SL + \gamma LG \cos \theta$$

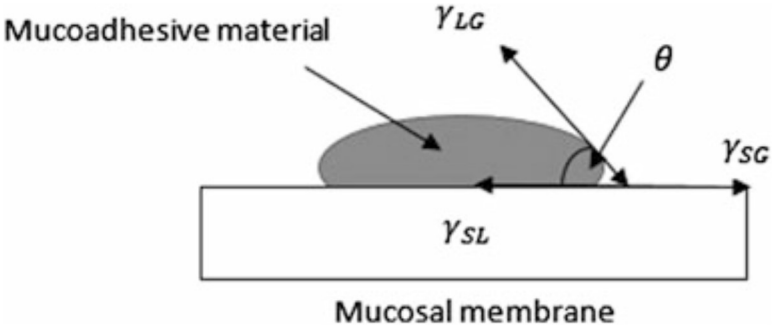


Figure 1.3- Contact angle measurement between a droplet and solid surface. (Reference: Wu et al, 2014)

Where γSG is the interfacial tension between solid and gas; γSL is the interfacial tension between solid and liquid; γLG is the interfacial tension between liquid and gas; and θ is the contact angle between solid and liquid interface. The interfacial tension associated with contact angle θ exhibits the degree of wetting.

When the contact angle θ is 0°, wetting is complete, the liquid having fully spread across the surface of the substrate. In contrast, a contact angle of 180° is indicative of no wettability.

1.6.1.3- Adsorption theory

The adsorption theory suggests that mucoadhesion is due to primary (ionic and covalent bonds) and secondary bond formation. The secondary (weaker) bonds such as Van der Waals forces, hydrogen bonding, electrostatic attraction, and hydrophobic interactions are the more desirable, resulting in semipermanent interactions that is an important criterion for drug delivery systems (Shaikh *et al*, 2011).

1.6.1.4- Diffusion theory

Diffusion theory describes that polymeric chains from the bioadhesive interpenetrate into glycoprotein mucin chains and reach a sufficient depth within the opposite matrix to allow formation of a semipermanent bond (Shaikh *et al*, 2011). The first step in this process involves the creation of an initial contact between the bioadhesive polymer chains and the mucus chains. In this step, weak physical forces, e.g., attraction and electronic force, dominate the mobility of the polymer chains. The second step involves the interpenetration of polymer chains from the delivery system into mucus layer to achieve mucoadhesion via more substantial bond formation. For significant interpenetration to occur, diffusion of the polymer chains of the dosage form into the mucin layer (and vice versa) must occur. Furthermore, the two components should have similar chemical structure to obtain the strongest mucoadhesive interaction (Wu *et al*, 2014).

1.6.2- Factors Affecting Mucoadhesion

Mucoadhesion may be affected by a number of factors, including hydrophilicity, molecular weight, cross-linking, swelling, pH value, and the concentration of the active polymer.

1.6.2.1- Hydrophilicity

Bioadhesive polymers possess numerous hydrophilic functional groups, such as hydroxyl and carboxyl. These groups allow hydrogen bonding with the substrate, swelling in aqueous media, thereby allowing maximal exposure of potential anchor sites. In addition, swollen polymers have the maximum distance between their chains leading to increased chain flexibility and efficient penetration of the substrate (Singh and Rana, 2012).

1.6.2.2- Molecular Weight

The interpenetration of polymer molecules is favoured by low-molecular-weight polymers, whereas entanglements are favoured at higher molecular weights. The optimum molecular weight for the maximum mucoadhesion depends on the type of polymer, with bioadhesive forces increasing with the molecular weight of the polymer up to 100,000(Shaikh, R *et al*, 2011).

1.6.2.3- Cross-linking and Swelling

Cross-link density is inversely proportional to the degree of swelling. The lower the cross-link density, the higher the flexibility and hydration rate, the larger the surface area of the polymer, the better the mucoadhesion. To achieve a high degree of swelling, a lightly cross-linked polymer is favoured. However, if too much moisture is present and the degree of swelling is too great, a slippy mucilage results and this can be easily removed from the substrate (Shaikh *et al*, 2011).

1.6.2.4- pH value

The pH value at the bioadhesive to substrate interface can influence the adhesion of bioadhesives possessing ionizable groups. Many bioadhesives used in drug delivery are polyanions possessing carboxylic acid functionalities. If the local pH value is above the p*K* of the polymer, it will be largely ionized; if the pH is below the p*K* of the polymer, it will be largely unionized. The approximate p*K* for the poly (acrylic acid) family of polymers is between 4 and 5. The maximum adhesive strength of these polymers is observed around pH 4–5 and decreases gradually above a pH of 6. A systematic investigation of the mechanisms of mucoadhesion clearly showed that the protonated carboxyl groups, rather than the ionized carboxyl groups, react with mucin molecules, presumably by the simultaneous formation of numerous hydrogen bond. (Park and Robinson, 1985)

1.6.2.5- Concentration of Active Polymer

In highly concentrated systems, beyond the optimum concentration the adhesive strength drops significantly. In concentrated solutions, the coiled molecules become solvent-poor and the chains available for interpenetration are not numerous. This result seems to be of interest only for more or less liquid mucoadhesive formulations.

Controlled drug delivery systems aim to deliver drugs at predetermined rates and predefined periods of time, targeting drugs to a desirable group of cells. In this perspective, nanoscale systems can maximize the efficacy of therapeutic treatments (Singh and Rana, 2012).

1.7- Options to overcome physiological barriers

Ocular drug absorption from the lacrimal fluid to the anterior ocular tissues via transcorneal absorption is determined by two major factors: drug permeability through the cornea and contact time of the product with ocular tissues. Based on these two principles, several approaches have been done to overcome the barriers.

One of the best strategies for improving the bioavailability of ocular drugs is to develop delivery systems that act as drug reservoirs, prolonging the residence time, controlling the release and, therefore, decreasing the frequency of administration (Almeida *et al*, 2014).

Colloidal carriers present several advantages for ophthalmic administration, like, small particle size, adhesive properties, improvement of the bioavailability of poorly water soluble drugs, protection of sensitive drug molecules (against enzyme inactivation), biodegradable, biocompatible and non-irritant features with corneal epithelial cells, improvement of drug pre-ocular retention, promoting absorption, targeted and controlled release characteristics, reducing or preventing side effects, being ideal for long-term treatments. (Mun *et al*, 2014).

Lipid and polymeric nanoparticles are the most promising for ophthalmic drug delivery and their use could revolutionize the therapy of many eye diseases. Nanoparticle systems are those containing particles with sizes smaller than 1000 nm,The smaller particles are better tolerated by patients than larger ones because they are better able to penetrate across the corneal barrier, where they will act as reservoir to release the drug slowly to the surrounding tissues (Ali and Al-Halafi, 2014).

1.7.1- Polymeric Nanoparticles

Biodegradable polymeric nanoparticle system, are the most popular systems in ocular carrier therapy. These systems can be composed of various polymers in which the drug is dissolved, entrapped or encapsulated. Drugs can either be integrated in the matrix or

attached to the surface. These systems offer several advantages for ophthalmic drug delivery, like biodegradability, non-toxicity, biocompatibility, mucoadhesiveness (bioadhesion and interaction with the glycoproteins of the cornea and conjunctiva), ease and low cost of production, and the possibility of obtaining stable systems after lyophilization and reconstitution, which increases the long-term stability of the systems (Almeida *et al*, 2014).

Polymers are classified as anionic, cationic and non-ionic (Singh and Rana, 2012). Cationic polymers have electrostatic interaction with the negatively charged epithelium present on mucosal surface. Mucoadhesion of cationic polymers, such as chitosan occurs due to the interactions of their hydroxyl and amino groups with the sialic groups of mucin in the mucus layer. Additionally, the linearity of chitosan molecules also ensures sufficient chain flexibility for interpenetration (Singh and Rana, 2012).

Chitosan, a (1–4)-2-amino 2-deoxy β-D-glucan, is a deacetylated form of chitin, which is the second-most abundant polymer in nature after cellulose (*Vyas et al,2012*).

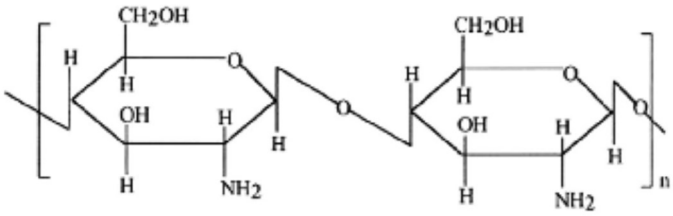


Figure 1.4-Schematic chemical structure of chitosan (Reference: Kunjachan et al, 2010)

Chitosan has one primary amino group and two free hydroxyl groups for each C6 building unit. Due to the availability of free amino groups, it carries a positive charge and reacts with many negatively charged surfaces such as the cell membrane, mucus lining (due to negatively charged sialic acid residues), and also with other anionic polymers (Kunjachan *et al*, 2010 and Rossi *et al* 2000).

It is considered a good candidate for ocular drug delivery because it is in the category of mucoadhesive polymers that are frequently used as an approach to prolong drug residence times. When using a mucoadhesive material, the clearance of the drug is controlled by the mucus turnover rate (approximately 15h to 20h), which is slower than the tear turnover rate. This prolonged retention of the drug formulation implies, for a drug with good permeability properties, an enhanced ocular drug bioavailability (Alonso and Sanchez, 2003). The mucoadhesive properties of chitosan in the eye are determined by the formation of either secondary chemical bonds such as hydrogen bonds or ionic

interactions between the positively charged amino groups of chitosan and the negatively charged sialic acid residues of mucins, depending on environmental pH (Ludwig, 2005). An alternative way to modulate the viscosity and viscoelastic behaviour of chitosan solutions could be through the incorporation of other hydrophilic polymers that are known to interact with chitosan like hyaluronic acid (Alonso and Sanchez, 2003).

Chitosan has penetration-enhancing properties, which are attributed to the modulation of the tight junction barrier between epithelial cells and also related to intracellular routes. Dodane *et al*, 1998 developed studies using Caco-2 cells and found that chitosan increases cell permeability by affecting both paracellular and intracellular pathways of epithelial cells in a reversible manner, without affecting cell viability or causing membrane wounds. This permeability-enhancing property has been used to explain the increased corneal transport of specific drugs. It is a biodegradable polymer, which enables the safe administration and degradation of topically applied ocular chitosan vehicles and its biodegradation is mediated by the hydrolytic actions of lysozyme and other enzymes (de la Fuente *et al*, 2010).

For ocular administration it is important to demonstrate that all the excipients used have low toxicity and to demonstrate this fact Felt *et al*. (1999), reported that chitosan has an excellent ocular tolerance. These results are based in the data observed in a rabbit model following topical instillation of chitosan solutions and using confocal laser scanning ophthalmoscopy combined with corneal fluorescein staining (Felt *et al*, 1999).

These studies provided evidence of the low irritation caused by chitosan after repeated topical administration to the corneal surface of rabbits (4 instillations a day for a period of 3 days). Similarly, an acute ocular tolerance test was also performed for chitosan coated poly-Ɛ-caprolactone (PCL) nanoparticles (Calvo *et al*, 1997). The results after repeated administration of these systems (2 instillations per hour for a period of 6 h), shown that no irritation or appreciable disruptions in the epithelial cells was observed.

In sum, chitosan is a good polymer to use due to its characteristics: easy of preparation, low cost, hypoallergenicity, non-toxicicity, biodegradability, do not interact with drugs.

Considering the chitosan advantages, a new type of nanosystem composed by chitosan as the main component was developed by Calvo *et al* 1997. Because of its cationic composition, chitosan is able to gel when in contact with specific multivalent polianions, such as sodium tripolyphosphate (TPP). Chitosan is a weak base, insoluble in water and organic solvents but it is soluble in dilute aqueous acidic solution (pH < 6.5), by converting the glucosamine units into a soluble form of protonated amine (R–NH3+).

Chitosan gets precipitated in alkaline solutions or with polyanions and forms a gel at a lower pH (Kunjachan *et al*, 2010).

Nanoparticles are spontaneously formed upon mixing of chitosan and TPP solutions, through the formation of inter and intramolecular linkages between the phosphate groups of TPP and the amino groups of chitosan. The method used is ionotropic gelation and it has been possible to incorporate efficiently hydrophilic compounds such as small molecules, peptides, proteins and genes through the establishment of electrostatic interactions either with the positively charged polymer chitosan or with the negative polyanion. The TPP is the main mechanism that governs the entrapment of these active compounds. The electrostatic interaction is particularly suited for the incorporation of biopharmaceuticals for two reasons: first, the formation process is solely based on the electrostatic interaction of oppositely charged polymers; hence, no chemical modification needs to be applied; secondly, the incorporation can be achieved in aqueous, physiological conditions (Agnihotri *et al*, 2004).

Besides natural polymers, synthetic mucoadhesive polymers like cellulose derivatives (e.g. cellulose-ethers: carboxymethyl cellulose (CMC) and hydroxypropylmethyl cellulose (HPMC) were also proposed as viscosifying agents. Cellulose-ethers have excellent biocompatibility profiles, stabilize the tear film and increase the contact time due to their film forming properties (Ludwig, 2005).

HPMC is a natural biodegradable and biocompatible anionic polymer obtained from natural cellulose by chemical modification, which empirical formula is $C_8H_{15}O_8$-$(C_{10}H_{18}O_6)_n$-$C_8H_{15}O_8$ and molecular weight is about 86000 ((Huichao *et al*, 2014). HPMC is selected for several formulations as a hydrophilic matrix system because it gives fast gel formation to control initial drug release and that the formation of its strong viscous gel controls further release. Its popularity can be attributed to its nontoxic nature, ease of compression, capability to accommodate a high level of drug loading, good viscosity stability between pH 3 and 11 during long-term storage (Huichao *et al*, 2014).

Hyaluronic acid (HA) is also incorporated in nanoparticles into the nanoparticles to improve their cellular targeting capacity. Although its characteristics of biocompatibility, biodegradability, and mucoadhesive character, HA is known for its implication in processes, as the regeneration of corneal and conjunctival epithelial cells, through its interaction with the CD44 receptor. CD44 is expressed in the human cornea and conjunctiva and participates in a wide variety of cellular functions, including receptor-

mediated internalization and degradation of hyaluronan (de la Fuente *et al*, 2008). It is because of this interaction with CD44 receptors that this system of nanoparticles is developed to penetrate the corneal and conjunctival epithelia (de la Fuente *et al*, 2010).

1.8- Bacterial keratitis

A wide diversity of eye diseases affects millions of people around the world and has devastating effects on individuals, leading to visual injury and possible ocular blindness that instigate a decline in quality of life (Karsten *et al*, 2012).

Bacterial keratitis is an infectious disease of the cornea that is characterised by inflammation, often with stromal infiltration by leukocytes, and is considered an ophthalmic emergency requiring immediate attention. Keratitis can progress rapidly with corneal destruction through pathological wound healing within 24-48 h (Karsten *et al*, 2012) that can result in severe visual loss and represents one of the most common causes of corneal blindness.

The common pathogens associated with the disease include *Staphylococcus aureus*, *coagulase-negative Staphylococcus*, *Pseudomonas aeruginosa*, *Streptococcus pneumonia,* and *Serratia* species, causing 90% of the bacterial keratitis cases (Karsten *et al*, 2012).

The most common keratitis risk factors are the use of contact lenses, corneal trauma, or underlying ocular surface disease.

The use of contact lenses has been closely associated with an increased incidence of bacterial keratitis. The introduction of soft contact lenses and their widespread use since the 1980s, is closely associated with an increased incidence of bacterial keratitis and knowing that 125 million worldwide them it is an important disease to control. Contact lenses can serve as a platform for the microbial proliferation, encouraging microbial adhesion to the cornea and can also interfere with the ocular defences, by leading to hypoxia and/or disruption of the epithelial-tear film interactions allowing the adhesion and invasion by the pathogen (Wong *et al*, 2012).

Direct corneal damage disrupts the innate immune system of the eye, enabling entry by microbes into the abrasion and consequent infection. Corneal surgery is a risk factor for microbial keratitis as the corneal epithelial barrier is breached. Due to the immune privileged nature of the eye, any break in the corneal epithelium renders the eye

susceptible to infection. Furthermore, post-operative care usually requires the use of topical steroids to control the immune response. The prophylactic use of corticosteroids has become quite controversial and has been associated with an increase in microbial growth and keratitis (Karsten *et al*, 2012).

1.9- Ceftazidime

Therapeutic failures to treat *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections of the eye with conventional regimens, as well as the emergence of resistant strains of these bacteria, have led to the use of fortified antibiotic eye drops in subjects who do not respond to conventional treatment modalities, as is often the case in long term care (Karampatakis *et al*, 2009).

Ceftazidime is a third-generation cephalosporin that acts by inhibition of bacterial cell wall synthesis. Ceftazidime has activity in the presence of some beta-lactamases, both penicillinases and cephalosporinases, of Gram-negative and Gram positive bacteria.

According to European Medicines Agency, ceftazidime has been shown to be active against most isolates of Gram-negative bacteria (*Citrobacter species, Enterobacter species, Esscherichia coli, Klebsiella species, Haemophilus influenza, Neisseria meningitidis, Proteus mirabilis, Proteus vulgaris, Pseudomonas aeruginosa, Serratia species)* and Gram-positive bacteria (S*taphylococcus aureus, Streptococcus pneumonia, Streptococcus pyogenes, Streptococcus agalactiae).*

2. Materials and Methods

2.1- Materials

Carboxymethyl cellulose (CMC) high and low viscosity, hydroxypropylmethyl cellulose (HPMC) low and high viscosity, phosphate buffer saline (PBS buffer), Chitosan low molecular weight (LMW), mucin from porcine stomach were obtained from Sigma–Aldrich (Dorset, UK).

Sodium tripolyphosphate (TPP) was obtained from Applichem (Germany). Hyaluronic acid (HA) (HA50, HA300 and HA3000 kDa) were kindly provided by Soliance (France) and ceftazidime (CFT) from Combino Pharm Portugal.

Tryptic soy broth (BK046HA) and Tryptic soy agar (15g/L agar-agar) were obtained from Biokar (France). *Pseudomonas aeruginosa* (ATCC® 9027), HEK293T (ATCC® CRL-11268™) and ARPE-19 (ATCC® CRL-2302™) cell lines were obtained from American Type Cell Culture collection (USA).

Cell culture media and supplements were from Gibco (ThermoFisher Scientific, UK).

Purified water was of Milli-RX quality (Merck Millipore, Germany). All other reagents and solvents were of the purest grade available, and used without further treatment.

2.2- Methods

2.2.1-Preparation of different polymeric solutions

The polymers used were carboxymethyl cellulose (CMC) of low and high viscosity and hydroxypropylmethyl cellulose (HPMC) of low and high viscosity at the percentages of 0.5%, 1% and 2% (w/V). The polymer powder was weighted according to the percentage desired and 20 mL of PBS was added to each sample and left to swell overnight.

The gel preparation was made in duplicate to evaluate the effect of autoclaving in the properties like pH value, viscosity and osmolality. The duplicated solutions were sterilized in the autoclave at 121ºC for 15 min.

2.2.1.1- Determination of viscosity

The viscosity measurements were carried out using a Brookfield viscometer model DVII (Brookfield, USA), using the spindle S8. The developed formulations were poured in the sampler tube using spindle nº S8. 10 mL of the gel sample was poured in the small sample

holder and viscosity measurements were taken at room temperature, every 30 s, at different shear rates (SR) in the range 0.5-100 rpm, and the spindle was lowered perpendicularly into it. For each sample, duplicate measurements were made, one at increasing SR and the other at decreasing SR, to account for time-dependent behavior.

2.2.1.2- pH value determination

For each gel formulation, the pH value was measured using a pH WTW meter (WTW GmbH, Germany) at room temperature. Equipment was previously calibrated using standard buffers of pH 4 and pH 7.

2.2.1.3- Osmolality determination

The osmolality of solutions was measured by the use of an Osmometer K-7400 (Knawer, Berlin, Germany). The equipment was previously calibrated at 0 and 300 mOsm/kg.

2.2.2-Preparation of the Nanoparticles

2.2.2.1- Formulation of CS/TPP/HA nanoparticles

The nanoparticles were produced by the ionotropic gelation method process based on the complexation between oppositely charged macromolecules. At this particular case, the positive charge of the hydrophilic polymer is complexed with a multivalent polyanion (sodium tripolyphosphate - TPP) to form highly viscous gel particles within the nanosize range. The basic mechanism involved in the formation of the nanoparticles is the electrostatic interactions between positively charged amino groups present in chitosan and negatively charged anion carboxyl and phosphate of HA and TPP previously described by Cadete *et al*. 2012.

At first, different amounts of CS (1 and 2.5 mg/mL) and TPP (2 and 3 mg/mL) at different pH were prepared. CS solutions were prepared by dissolving the polymer in purified water with 1% (V/V) acetic acid solution and leaving it under stirring for 3 h. The pH value was adjusted to 4, 5 and 6 with a 0.1N sodium hydroxide solution. TPP solutions were prepared by dissolving TPP in purified water, and the pH was adjusted to 7.5, 8 and 9.

For the production of nanoparticles the proportion of the three components CS, HA and TPP, the pH value and the molecular weight of HA had to be optimized. HA was used at three different molecular mass, 50, 300 and 3 kDa at the concentration of 10 mg/mL. The

assembling of CS an TPP in different concentrations and pH values allows the analysis of many formulations with different proportions TPP:CS, from 33:1 to 4:1.
The HA, at different molecular mass, was added to TPP solution and suffered successive dilutions. This mixture was then added to CS solution. The yield of the different formulations was measured by optical density (OD) by measuring the samples absorbance at a wavelength of 600 nm (FLUOstar Omega, BMG Labtech, Germany).

2.2.2.2- Particle size distribution

Mean particle size and polydispersity index (PDI) of nanoparticles were assessed by light scattering using a Zetasizer Nano-S (Malvern Instruments, UK) at a temperature of 25ºC. Nanoparticles were dispersed in 0.22 μm filtered purified water and their distribution size was obtained from the average of three different batches.

2.2.2.3-Surface charge evaluation

The surface charge of the nanoparticles was evaluated through zeta-potential measurement at Malvern Zetasizer Nano-Z (Malvern Instruments, UK). Zeta-potential is based on dynamic light scattering methodologies while applying an electrical field that will polarize an electrophoretic cell and the surface charges will be evaluated due to electrophoretic mobility/velocity in the cell through the correlation equation of Smoluchowski which relates the velocity of the molecule through the electrical field with its charge (Domingues et al., 2009).
Standard electrophoretic cell was used with gold electrodes to apply the electric field. Samples were diluted in 3 mL of 0.22 μm filtered purified water and the cell was filled verifying for the existence of bubbles that could cause interference in the zeta-potential measurements. All experiments were done in triplicate.

2.2.2.4- Scale up of selected nanoparticles formulation

The formulation with better characteristics of OD, size and zeta-potential was chosen to be scaled-up for 1 mL and 10 mL and formulated as empty nanoparticles and nanoparticles loaded with ceftazidime.
The volumes of the solutions used to prepare a solution of NP with 1 mL and 10 mL are presented in table 2.1. The 1mL NP solutions were prepared to be used for the encapsulation efficiency test. To evaluate if the method of production of the NP solutions can be scaled up, the 1mL NP solution was scaled up 10 times.

150 µL of the mix solution of TPP-HA-(CFT) was added to 1 mL of the CS solution and the homogenization was performed by pipetting several times. An opalescent suspension was immediately obtained. For the solution of 10 mL, the preparation was similar but the addition of TPP-HA-(CFT) to chitosan solution was made under constant stirring (300 rpm).

After choosing the best nanoparticles formulation, the different concentrations of ceftazidime were tested (50, 100, 150 and 200 µg/mL). All the formulations were tested in triplicate.

Table 2.1- Composition of nanoparticles solutions

Solutions	**Volume (µL)**					**Volume (mL)**	
	Empty NPs	**NPs loaded with CFT**				**Empty NPs**	**NPs loaded with CFT**
CS (1 mg/mL)	1000	1000				10	10
TPP (3mg/mL)	75	75				0.75	0.75
HA 50 (10mg/mL)	19	19				0.19	0.19
CFT (10mg/mL)	-	5	10	15	20	-	0.2
H_2O	56	115	110	105	100	0.56	0.36
	Total: 1.150ml					**Total: 11.50 ml**	

2.2.2.5- Nanoparticles production yield

Chitosan concentration was determined using an indirect method based on the quantification of the chitosan concentration initially used in the formulation, and that found in the supernatant of the final nanoparticle suspension. The method for chitosan quantification was based on a colorimetric reaction between the amine groups of chitosan and the dye Cibacron brilliant red 3B-A, adapted to a 96-well microplate (Cadete et al., 2012). Briefly, 1 mL of the nanoparticles suspension was centrifuged (Sigma 112,

Germany) at 12000×g for 15 min at room temperature in order to separate the nanoparticles from free chitosan.

After the addition of the dye to the solutions the absorbance values were measured at 575 nm in FLUOstar Omega (BMG Labtech, Germany). Standard curve was created using a known range of chitosan concentrations in the same conditions as the unknown samples. Unknown chitosan concentration in each sample was determined using linear regression analysis. Thus, it was possible to determine the chitosan yield (CS yield) using the following equation:

$$\text{CS yield}(\%) = \frac{[\text{CS}]\text{total} - [\text{CS}]\text{supernatant}}{[CS]total} \times 100$$

[CS]total is the concentration of chitosan used to prepare the nanoparticles and [CS] supernatant is the concentration of chitosan in the supernatant.

2.2.2.6-Quantification of Ceftazidime

The quantification was assessed by UV-Visible spectrophotometry, at λmax of 256 nm, in a microplate spectrophotometer reader (FLUOstar Omega, BMGLabtech, Germany). Calibration curves with standard concentrations in the range of 250-1.95 µg/mL were used.

2.2.2.7- Encapsulation efficiency

Encapsulation efficiency (EE) was determined by quantification of CFT in the supernatants (i.e., non-encapsulated CFT) obtained during nanoparticles preparation. Antibiotic detection was performed as previously described (2.2.2.6.) The EE is expressed as the percentage of antibiotic encapsulated in particles reported to the initial amount of antibiotic used for particle preparation. The encapsulation efficiency (EE) of the ceftazidime was determined by subtracting the free drug amount from the initial added amount of the drug. The (EE %) was calculated by the following equation:

$$\text{EE } (\%) = \frac{[\text{inicial CFT}] - [\text{supernatant CFT}]}{[\text{initial CFT}]} \times 100$$

2.2.3- Bioadhesion studies

The bioadhesion was evaluated by viscosity and zeta potential measurements.

The viscosities of the polymeric solutions were determined at room temperature by using the Ostwald viscometer, by means of the following equation:

$$\eta 1 = \eta 2.\rho 1 t 1/\rho 2 t 2$$

Where η1 and η2 are viscosity coefficients of our solution and water, ρ1 and ρ2 are the densities of the solution and water, and t1 and t2 are the flow times measured in the viscometer of the solution and water, respectively.

The viscosities of HPMC 0.5% high viscosity, mucin and nanoparticles were measured. At first, the viscosity of each individual component was determined and after that, to evaluate the effect of mucin interaction in the solutions two sets of samples were prepared: 1) mucin was added to the solutions of HPMC 0.5%, empty NP and empty NP gel; 2) the same study was developed with ceftazidime nanoparticles.

The viscosity component due to bioadhesion or rheological synergism parameter was obtained with the equation (Hassan and Gallo, 1990):

$$\eta_t = \eta_m + \eta_p\ \eta_b$$

Where η_t is the viscosity coefficient of the system, and η_m and η_p are the individual viscosity coefficients of mucin and bioadhesive polymer, respectively. ηb is the viscosity component due to bioadhesion.

Another method to evaluate the nanoparticles-mucin interaction was determined by measuring the zeta potential of the mixtures of mucin and particles system using a Zetasizer Nanoseries Nano Z (Malvern Instruments, Malvern, UK).

2.2.4- *In vitro* ceftazidime release studies

The ceftazidime release from nanoparticles involved an *in vitro* assay using pre-assembled dialysis device membrane (Float-A-Lyzer G2, pore size cut-off of 100 kDa, Spectrum Labs, Germany).

The samples analysed were: empty nanoparticles, empty nanoparticles with HPMC, ceftazidime nanoparticles and ceftazidime nanoparticles with HPMC. Suspensions with empty nanoparticles were used as control. Five samples of the suspensions of ceftazidime nanoparticles were analysed.

To each membrane 1 mL of nanoparticles suspension was added and 15 mL of the receptor phase composed by phosphate-buffered saline (PBS) pH 7.4. The system was kept in agitation (300 rpm) in a shaker (Labinco LD-40, Netherlands) at 37ºC during 24h. At pre-determined intervals (0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6 h) (200 µL of the receptor phase was collected to a 96 well-plate). The withdrawn aliquots were always replaced with equal volume of PBS pH 7.4, maintained at the same temperature. The released ceftazidime was quantified by the technique previously described (2.2.2.6).

In order to investigate the mechanism of ceftazidime release from the nanoparticles and the gel, the release data were fitted to different kinetic models including: zero-order, first-order, Higuchi, and Korsmeyer–Peppas (Costa and Lobo, 2001).

2.2.5- *In vitro* ceftazidime permeation studies

Franz cells are largely used for *in vitro* permeation studies because of the simplicity in the use. The diffusion cells used were a system of two compartments, donor and receptor, separate by a membrane (Karn, 2014)

The receptor compartment has an approximate volume of 5 mL and a diffusion area of 1 cm^2. The membranes used in the diffusion assay were Tuffryn® synthetic membranes made of hydrophilic polysulfone, with 0.45 µm of porosity. The samples were then applied (0.192 g ± 0.1 g) on the surface of the membrane in the donor compartment and immediately sealed with Parafilm® to prevent evaporation.

The receptor compartments were filled with approximately 5mL of PBS buffer pH 7.4, with all air bubbles expelled from the compartment. The weight of each one was determined. Aliquots (200 µL) of the samples were added to the donor compartment and the weight was also measured. All the systems were maintained at the temperature of 37ºC with constant stirring (300 rpm) using a magnetic bar.

The permeation study was carried out for 6 h. During the first hour the samples were collected at each fifteen minutes and after that time at each hour. At each determined time, 200 µL of the receptor phase was collected to a 96 well-plate. The withdrawn aliquots were always replaced with equal volume of PBS pH 7.4, maintained at the same temperature. All experiments were carried out using 5 cells per formulation and performed under sink conditions during the whole experiment. Samples were analysed for ceftazidime content by the previously described technique (2.2.2.6).

2.2.6- Microbiological assay

2.2.6.1- Agar diffusion method

Tryptic soy agar was used as the growth medium, with a concentration of 15g/L agar-agar, this solution was sterilized in an autoclave at 121ºC for 15 min. Under aseptic conditions, the Tryptic soy agar containing the microorganism *Pseudomonas aeruginosa* (ATCC 9027) was placed in a Petri dish and solidified. Paper discs were positioned on the solid agar. The samples of free CFT, empty CS/TPP/HA NP, and empty NP in HPMC gel and CS/TPP/HA NP in gel were placed in the paper discs in triplicate. The volumes used were 15, 7.5 and 5 µL, three paper discs for each sample were considered. To evaluate a possible loss in the antimicrobial activity with time, samples of CS/TPP/HA NP with 1 day and with 2 months were also placed in the paper discs in triplicate. The Petri dishes were incubated at 35 ± 2°C overnight. In this assay the parameter used to study the antimicrobial activity was the mean diameter of the inhibition zone formed around the disc, after incubation. The diameters were measured using a Vernier caliper.

2.2.6.2- Microtitre plate antibacterial assay

This microtitre plate antibacterial assay was adapted from the method previously described according to the guidelines of Clinical and Laboratory Standards Institute (CLSI), 2012 by broth microdilution method. To perform this assay the medium used was Tryptic soy broth (TSB) (BK046, Biokar, France) which allows the growth of a large number of species including the more fastidious like *P. aeruginosa*. The dehydrated medium was reconstituted with purified water to obtain a final concentration of 30g/L, after stirring until complete dissolution the tubes containing the medium were sterilized in an autoclave at 121ºC for 15 min. Under aseptic techniques a colony of microorganism *P. aeruginosa* was transferred into a bottle containing the TSB medium, the bottle was capped and incubated overnight at 35 ± 2°C. The optical density of *P. aeruginosa* was recorded at 600 nm, and various dilutions were made with aseptic techniques until the optical density was between 0.5-1.0. The dilution was carried out to achieve a concentration of 1×10^6 cfu/mL. To evaluate the possible differences in the antimicrobial effect of CS/TPP/HA NP prepared in different times, we used in this assay NP with 1 day and 2 months, in addition the antimicrobial effect of NP in gel and of free CFT was evaluated. The final concentration of free CFT and CFT in NP used in this assay was 100µg/mL.

The plate was prepared under aseptic conditions; a sterile 96 well plate was used. The NP samples and free CFT were subject to serial dilutions. Finally, the 50µL of 1×10^6 cfu/mL bacterial suspension was added to each well to achieve a concentration of 5×10^5 cfu/mL. The plate was incubated at 35±2°C overnight. The turbidity change was first assessed visually. A vital dye, resazurin (Alamar Blue), was added to all wells in the plate and incubated for 3h. To confirm the positive and negative results the wells fluorescence intensity was measured in a microplate spectrophotometer reader (FLUOstar Omega, BMGLabtech, Germany). The first concentration with no sign of bacterial growth (sample with low fluorescence intensity) was taken as the MIC (minimum inhibitory concentration) value.

2.2.7- *In vitro* cell assays

2.2.7.1- Cell viability

The cytotoxicity was assessed using general cell viability endpoint resazurin reduction (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) (Alamar Blue) assay (Ferreira *et al*, 2015, Nociari *et al*, 1998)and propidium iodide (PI) dye exclusion assays (Mehanna *et al*, 2011). The resazurin is a blue dye, weakly fluorescent that is reduced by viable cells into the pink colored and highly red fluorescent resorufin. The propidium iodide (PI) is a red fluorescent probe that is a cell membrane impermeant and, therefore, only penetrates membrane integrity compromised cells. When PI does gain access to nucleic acids, and intercalates them, its fluorescence increases dramatically and it is, therefore, used to identify membrane integrity compromised cells.

Cell viability was assessed after 24h of incubation with different concentrations of the formulations. The day before experiment HEK293T (human embryonic kidney cell line, ATCC® CRL-11268™) and ARPE-19 (human retinal pigment epithelial cell line, ATCC® CRL-2302™) cell lines were seeded in sterile flat bottom 96 well tissue culture plates (Greiner, Germany), in RPMI 1640 culture medium (Life Technologies, UK), supplemented with 10% Fetal serum bovine (Life Technologies, UK), 100 units/mL of penicillin G (sodium salt) (Life Technologies, UK), 100 µg/mL of streptomycin sulfate (Life Technologies, UK), 2mM L-glutamine (Life Technologies, UK), at a cell density of 2×10^5 cells/mL. Cells were incubated at 37ºC and 5% CO2.

On the next day, medium was replaced by fresh medium containing the different samples to be analyzed. Each concentration was tested in six wells per plate. Cells were incubated for 48h, negative control was the culture medium and positive control sodium dodecyl

sulfate (SDS) at 0.1 mg/mL. After the time of exposition, the medium was replaced by 0.3 mM propidium iodide in culture medium (stock solution 1.5 mM in DMSO, diluted with culture medium 1:5000). Fluorescence was measured (excitation, 485 nm; emission, 590 nm) in microplate reader (FLUOstar Omega, BMGLabtech, Germany), and then, the Alamar Blue assay was performed. Medium was replace by medium containing 5 mM of resazurin. The cells were further incubated for 3h and the fluorescence at 530 nm of excitation wavelength and 590 nm of emission wavelength was measured in a fluorescence microplate reader (FLUOstar Omega, BMGLabtech, Germany).

The relative cell viability (%) compared to control cells was calculated by [Fluorescence]sample/[Fluorescence]control×100 for the Alamar Blue assay and [Fluorescence]sample/[Fluorescence]control for PI uptake assay.

2.2.7.2- Oxidative stress assay

The intracellular reactive oxygen species (ROS) production was determined using the 2-7'dichlorodihydrofluorescein diacetate (H2-DCFDA) dye. DCFH-DA is a stable, non-fluorescent molecule that is hydrolyzed by intracellular esterases to non-fluorescent 2-7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized in the presence of oxygen radicals to a highly fluorescent compound (DCF) (Crow, 1997). Cultures at same cell density for cell viability assay (2.2.7.1) of ARPE-19 and HEK 293T cells grown in 96-well plates were incubated, after exposed for 24h at different samples, 30 min with 20 μM of H2-DCFDA (Life Technologies, UK) in the dark at 37ºC. The medium was then removed and fresh medium was added. Hydrogen peroxide solution (Applichem, Germany) was used as a positive control for the induction of ROS in cells and media alone as a negative control. ROS levels were determined at excitation 485 nm and emission 520 nm wavelengths using a florescence microplate reader (FLUOstar BMGLabtech, Germany). Data from 6 replicates are reported as relative fluorescence units (RFU) percentage and expressed as mean fluorescence ratio (fluorescence of exposed cells/fluorescence of unexposed control from the same experiment) (Crow, 1997).

2.2.8- Statistical Data Analysis

The data was expressed as mean and standard deviation (mean ± SD) of experiments. Statistical evaluation of data was performed using one-way analysis of variance (ANOVA). Tukey–Kramer multiple comparison test (GraphPad PRISM 5

software, USA), was used to compare the significance of the difference between the groups, a $P < 0.05$ was accepted as significant.

3. Results and Discussion

3.1 Preparation of the Nanoparticles

The nanoparticles were produced by the ionotropic gelation method process based on the complexation between oppositely charged macromolecules.

At first, different amounts of CS (1 and 2.5 mg/mL) and pH values of 4, 5 and 6 and TPP (2 and 3 mg/mL) at different pH values 7, 8 and 9 were tested.

For the production of the nanoparticles it has to be optimized the proportion of the three components CS, HA and TPP, the pH value and the molecular weight of HA. HA was used at three different molecular mass, 50, 300 and 3000 kDa at the concentration of 10 mg/mL. The assembling of CS and TPP in different concentrations and pH allows the analysis of many formulations with different weight proportions CS:TPP:, from 33:1 to 4:1. The yield of the different formulations was measured by optical density (OD) measuring the samples absorbance at a wavelength of 600 nm.

With the solution of CS 2.5mg/mL and for the two tested concentrations of TPP solution and pH values containing 5mg/mL of HA 50kDa values present aggregates (table 3.1).

Table 3.1. Screening of the preparation of the nanoparticles for different concentrations of TPP at fixed concentration of HA 50kDa of 5mg/mL and two concentrations of CS and three pH values.

	CS 2.5 mg/mL			CS 1 mg/mL		
	pH 4	pH 5	pH 6	pH 4	pH 5	pH 6
TPP 2 mg/mL pH 7 +AH 50kDa	A	A	A	NT	T	T
TPP 2 mg/mL pH 8+AH 50kDa	A	A	A	NT	T	T
TPP 2 mg/mL pH 9+AH 50kDa	A	A	A	NT	T	T
TPP 3 mg/mL pH 7+AH 50kDa	A	A	A	NT	T	T
TPP 3 mg/mL pH 8+AH 50kDa	A	A	A	NT	T	T
TPP 3 mg/mL pH 9+AH 50kDa	A	A	A	NT	T	T

A-aggregates; NT-not turbid; T-turbid (Ratio volume of TPP/HA to CS was 1:7)

Thus further studies were performed with CS at 1mg/mL for the pH values of 5 and 6 (table 3.2).

Table 3.2. Screening of the preparation of the nanoparticles for two concentrations and at two pH values of TPP with different concentrations of HA 50kDa, 300kDa and 3000kDa with 1 mg/mL CS and two pH values.

	CS 1 mg/mL	
	pH 5	pH 6
TPP 2 mg/mL pH 8+AH 50kDa	T	T
TPP 2 mg/mL pH 9+AH 50kDa	T	T
TPP 3 mg/mL pH 8+AH 50kDa	T	T*
TPP 3 mg/mL pH 9+AH 50kDa	T*	T*
TPP 2 mg/mL pH 8+AH 300kDa	A	A
TPP 2 mg/mL pH 9+AH 300kDa	A	A
TPP 3 mg/mL pH 8+AH 300kDa	A	A
TPP 3 mg/mL pH 9+AH 300kDa	A	A
TPP 2 mg/mL pH 8+AH 3000kDa	A	A
TPP 2 mg/mL pH 9+AH 3000kDa	A	A
TPP 3 mg/mL pH 8+AH 3000kDa	A	A
TPP 3 mg/mL pH 9+AH 3000kDa	A	A
A-aggregates; T-turbid; T*- Particles size >2000nm (Ratio volume of TPP/HA to CS was 1:7)		

The concentration of HA was changed on the range of 10mg/mL to 0.04mg/mL and it was possible to observed that for high molecular mass 300kDa and 3000kDa or the particles aggregates or the size was higher than 1000nm, in all the tested conditions.
The chosen formulation to proceed was the one with a considerable yield of production and size range lower than 1000 μm (table 3.3).

Table 3.3- Composition of the selected nanoparticles (size, polydispersity index (PdI) and Zeta potential **(**mean±SD, n=3)

	Volume (µL)	Size (nm)/PdI	Zeta Potential (mV)
CS (1mg/mL, pH 5)	1000	795±14	+40±1
TPP (3mg/mL. pH 7.0)	75	0.363±0.073	
HA 50 (10 mg/mL)	19		
H_2O	56		

3.2 Selection of adequate polymer to contain CS/HA nanoparticles

The main purpose of the developed work was the development of an effective ceftazidime carrier system to be used to deliver the antibiotic directly into the eye. It is an innovation way to apply ceftazidime because nowadays this antibiotic is only used for intravenous treatments.

CS/HA nanoparticles were selected for ocular delivery, due to chemical and biological properties of hyaluronic acid and chitosan, such as biocompatibility, biodegradability, mucoadhesiveness and non-toxicity, as described previously (Kunjachan *et al*, 2010). Nanoparticles are carrier systems that must allow improving the efficacy of drug delivery by overcoming diffusion barriers, permitting reduce doses as well as sustained delivery and appear to be the most promising tool to meet the primary requirements of and ideal ocular delivery system (Almeida *et al*, 2014).

The most important aspect in the development of eye drops is the compatibility with the ocular mucosa. All ocular formulations must ensure isotonicity (osmolality close to 300 mOsm/kg), pH similar to the physiological fluids (pH 7.4), optimal viscosity and sterility (Almeida *et al*, 2014)

To produce nanoparticles ocular instillation, two different polymers to incorporate the nanoparticles were studied: HPMC and CMC high and low viscosity. The two polymers were tested in three different concentrations 0.5, 1 and 2% (w/v). Because ophthalmic preparations have to be sterile the first test was to verify if the polymer maintained the

same characteristics before and after sterilization by autoclave (121ºC for 15 minutes). The determination of gels viscosity is important because it must have the correct viscosity that allows to increase the ocular contact time increasing de mucoadhesiveness and with this increase bioavailability. It cannot be so fluid that have a rapid drainage and not so viscous that cause blurred vision (Zambito and Colo, 2011)

Both HPMC and CMC 0.5, 1 and 2% (w/v) have a Newtonian behavior and the viscosity of the gels, autoclaved and not autoclaved, is very similar when comparing the same polymer solution with equal concentration (data not shown).This means that autoclaving does not interfere with the rheology of the gel. By analyzing the viscosity values (table 3.4) obtained for all the tested polymers the ones that were selected to proceed with other tests were: HPMC 1%(w/v) low viscosity, HPMC 0.5%(w/v) high viscosity, CMC l% (w/v) low viscosity and CMC 0.5% (w/v).

Table 3.4- Determination of the gel viscosity

Solution (w/v)	Viscosity Autoclaved gel (mPa.s)	Viscosity non autoclaved gel (mPa.s)
HPMC	54.5	57
	174	170
	210	300
CMC	85	100
	88,5	95
	Non-Newtonian fluid	

3.3 Determination of pH value and Osmolality

The selected polymer concentrations were evaluated according to their pH and Osmolality to ensure that they are compatible with the ocular mucosa. The European Pharmacopoeia 8th, the monograph “Ophthalmic Preparations” indicates that the value of pH 7.4 and osmolality around 300 mOsm/kg correspond to an isotonic solution.

The results obtained (table 3.5) show that autoclaving does not affect pH and osmolality values, being an advantage because sterilization does not interfere with the desired characteristics for the final formulation. The evaluated polymers can ensure isotonic formulations allowing that any of them can be used in the final formulation of the eye drops.

Table 3.5 - Determination of pH value and Osmolality

Solution (w/v)	pH value		Osmolality (mOsm/ Kg)	
	Autoclaved gel	Non autoclaved gel	Autoclaved gel	Non autoclaved gel
HPMC LV 1%	7.34	7.34	260	270
HPMC HV 0.5%	7.36	7.35	254	271
CMC LV 1%	7.35	7.35	266	267
CMC HV 0,5%	7.35	7.30	261	268

3.4 Determination of Zeta Potential

Results in table 3.6 show that autoclaving does not affect significantly ZP values, being once again the advantage to achieve a sterile formulation without affecting its properties.

Table 3.6 - Zeta potential (ZP) values of the tested polymeric solutions (mean±SD, n=3)

Solution (w/v)	ZP (mV)	
	Autoclaved gel	Non autoclaved gel
HPMC LV 1%	-2±0	-11±2
HPMC HV 0.5%	-3±2	-9±5
CMC LV 1%	-26±103	-31±4
CMC HV 0.5%	-24±5	-31±3

The ZP values demonstrate that CMC and HPMC solutions have negative values but with significant differences between them. A possible explanation for this difference could be that HPMC has a nonionic nature while CMC is anionic, which makes its surface charge more negative (Sosnik *et al*,2014). The distribution of the ZP values is shown on figures 3.1 and 3.2.

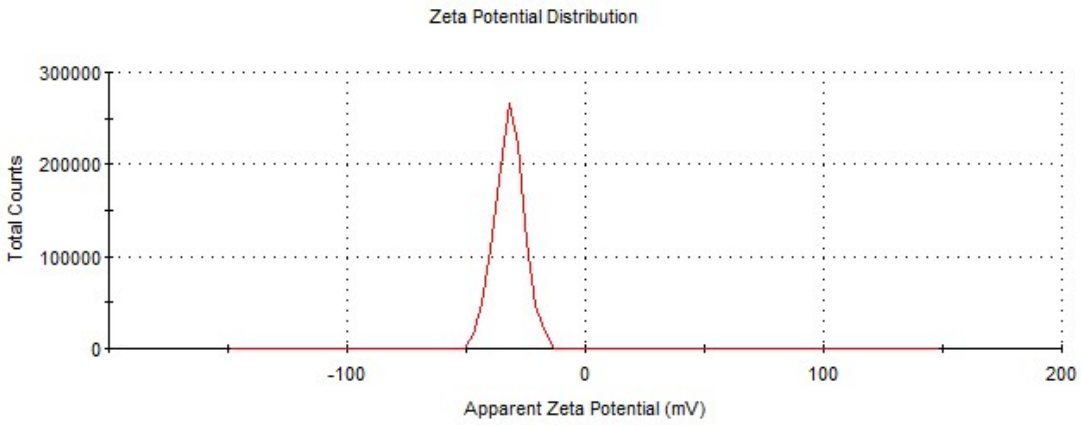


Figure 3.1 - Distribution of ZP in CMC

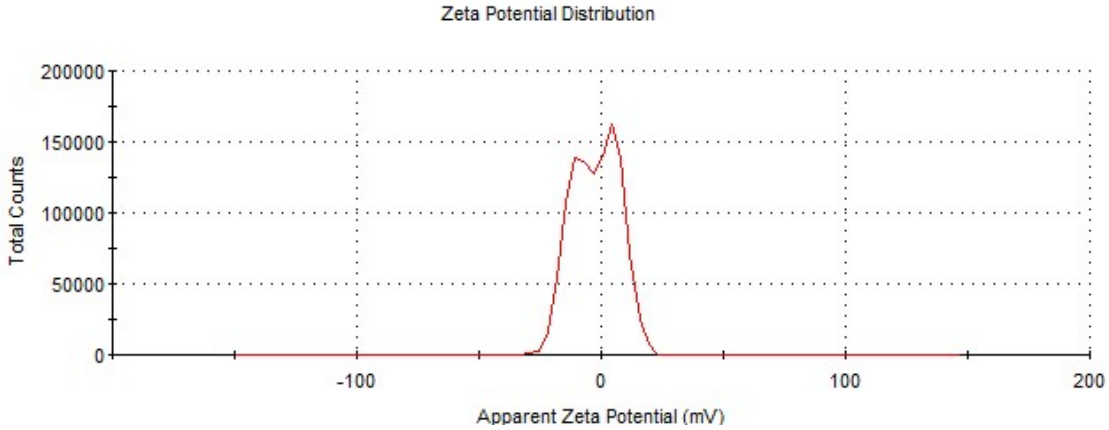


Figure 3.2 - Distribution of ZP in HPMC

After the characterization of the polymers (pH, osmolality and ZP), it is concluded that both could be used to prepare two different eye drop formulations to increase CFT absorption and bioavailability. However, after combining CS/TPP/HA it was observed that with CMC the nanoparticles precipitated instead of what happened with HPMC that maintained nanoparticles stable in solution (Figure 3.3). CMC has a negative charge and for this reason it interacts with the positive charges of CS/TPP/HA nanoparticles resulting in flocculation of the nanoparticles. In contact with mucin, it has been described that the interaction of CMC with mucin is good only at low pH value. When the pH increases the adhesive interactions are practically absent; a possible explanation could be the fact that the pKa of CMC is near 3.5, which means that above this value the polymer is negatively

charged leading to the establishment of repulsive forces with the negative charges from the mucin chains (Sosnik *et al*, 2014).

In its turn HPMC has a charge near zero and for this reason it allows nanoparticles to be maintained in suspension. For this reason, HPMC was the selected polymer to contain the CFT nanoparticles. In fact, HPMC is largely used in pharmaceutical formulations as lubricant for the treatment of the Dry Eye Syndrome (Zambito and Colo, 2011).

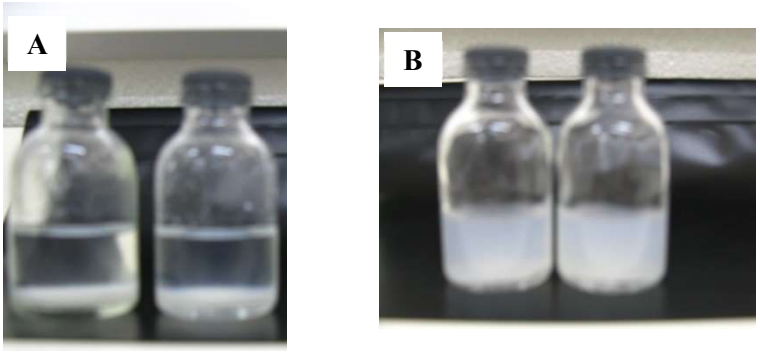


Figure 3.3– Incorporation of ceftazidime nanoparticles in CMC 0.5% and 1% (w/V) polymer (A) and HPMC 0.5% and 1% (w/V) (B)

3.5 Nanoparticles encapsulating CFT

Because CFT suffers degradation in aqueous solutions, it was necessary to develop a different system to make the topical ocular administration. NP was the developed system to interact with the mucosal barrier with the objective of favoring the transport of macromolecules into the ocular mucosa. Developed polymeric NP represent an approach as they can entrap macromolecules and protect them against degradation and are able to increase ocular penetration and bioavailability due to their small particle size (Bravo-Osuna, 2016). The use of mucoadhesive polymers allows the interaction with the mucus layer increasing the biopharmaceutical residence and release time. Natural bioadhesive polysaccharides like HA and CS are particularly interesting for the development of polymeric NP intended for ophthalmic administration. The association of these two polymers is intended to promote the utility of the nanocarriers for the ocular delivery of macromolecules (Singh and Rana, 2012).

The mechanism of formation of these NP is believed to combine the hydrogen bonds and electrostatic interaction between CS and HA (that are oppositely charged) with the ability of CS to gel spontaneously due to its ionic interaction with polyanion TPP. The addition of the TPP crosslinker is responsible for the formation of very organized structures, due to the reticulation of the nanosystems, TPP is a non-toxic molecule which can form a gel by ionic interactions between the positively charged amino groups of CS and the multivalent anions of TPP (de la Fuente *et al*, 2008). Because ceftazidime has a polar character (logP= -1.6), its encapsulation in CS/TPP/HA NP is probably achieved through hydrogen bonds and electrostatic interactions (Wijesooriya, 2013).
The developed CFT nanoparticles maintained the same characteristics of the empty NPs as shown in table 3.7

Table 3.7 – Determination of NP´s size, polydispersity index (PdI) and Zeta potential (mean±SD, n=3)

	CFT (µg/mL)	**Size (nm)**	**PdI**	**ZP (mV)**
Empty NP	0	795±14	0.363±0.073	+40±1
CFT NP	50	632±11	0.280±0.010	+37±1
	100	743±7	0.360±0.070	+38±0
	150	675±11	0.290±0.040	+39±0
	200	654±4	0.300±0.050	+37±1

The obtained NP has the required characteristics: high and positive zeta potential to provide electrostatic stabilization, the dimensions in the range of 100-1000nm and narrow size dispersity.
The obtained size of CFT NP is adequate to ocular administration in eye drops and it is easily transported across biological barriers.
The polydispersity index (PDI) smaller than 0.4 indicates a relative homogeneous dispersion. The positive ZP values indicate that the surface of the NP is mostly composed by CS. The positive charge of the particles is desirable to prevent particle aggregation and promote electrostatic interaction with the negatively charged sialic acid residues of mucin in the eye surface (Singh and Rana, 2012). The ZP value higher than ±30mV indicates a nanosuspension stable by electrostatic repulsion which leads to a more uniform size

distribution. The stability of the particles is important to prevent their aggregation (Gonçalves *et al*, 2010).

The scale-up of NP formulation ten times, to a final volume of 10mL, was accomplished and can be verified that their characteristics are similar to the NP 1mL samples. This information is important to prepare a CFT eye drop formulation that could be commercially manufactured for the treatment of eye infections. The main factor affecting the CFT association to the NP is probably electrostatic interactions. CFT is in a HA/TPP solution with a basic pH, consequently its charge is mostly negative which enhances CFT interactions with the free amino groups present in the CS acidic solution (de la Fuente *et al*, 2008).

Wijesooriya *et al.* (2013), developed a liposomal formulation containing CFT, when using a cationic lipid DOTMA to encapsulate CFT. In the study it was observed a decrease in the EE. The explanation for this result was the electrostatic interactions between the lipid bilayer and the drug that negatively influenced the rate of encapsulation due to their positive charges. This explanation seems reasonable as our results confirm that when CFT is added directly to the CS solution with pH=5, where CS has also a cationic nature, the EE is lower than when CFT is added to mix solution. The positively charged quaternary ammonium of CFT may play a significant role in the aforementioned fact. Thus, the addition of CFT to the solution of TPP/HA/H_2O mix, that has a higher pH value, allow CFT to be negatively charged which promotes the interaction with CS enabling a more efficient CFT encapsulation in the NP. However, we managed to have higher encapsulation efficiencies with CS/TPP/HA NP than with liposomes. This may have occurred because the LMW chitosan is a small molecule (MW: 50000-190000 Da) comparing to the cationic lipid DOTMA (MW: 670575 Da), so it is possible that in liposomes the effect of electrostatic interactions have a stronger effect in the encapsulation of CFT.

The Encapsulation Efficiency (table 3.8) determined as indicated in section 2.2.2.7, shows a high percentage of encapsulated CFT, allowing to conclude that the developed process is highly effective for the maximum tested concentration.

Table 3.8- Determination of ceftazidime Encapsulation efficiency (EE) (mean±SD, n=12)

	EE (%)
NP CFT (200µg/mL)	78.4± 1.0

3.6 *In vitro* ceftazidime release studies

For the NP in solution, 50% of the dug was released after 3h, while for the NP included in the HPMC, at the same time, 30% of CFT was released (figure 3.4.). These values remained stable over 24h (data not shown). The drug release from the NP seemed to have two different stages, an initial fast release of ≈25% for the NP+HPMC gel and ≈40% in the NP in solution until the first hour (figure 3.4). This was followed by a slower exponential release of the remaining drug over the next hours. The initial rapid release, known as "burst effect", occurs due to the fact that some of CFT were adsorbed on the surface of NP which could be released easily by diffusion, additionally there are amounts of CFT that were not encapsulated and remained on solution and so they have a fast dissolution in the release medium. After the initial burst effect, a slower sustained release occurs which can be attributed to the slow diffusion of the encapsulated drug out of the polymeric matrix of NP into the release medium. These release profiles support the idea that some CFT molecules were encapsulated among the positively charged hydrophilic chains of CS and the remaining molecules were either adsorbed to the NP surface or free in solution. The HPMC gel is another possible barrier to the drug release, with lower values for drug dissolution in the release medium. The gel entraps the CFT molecules to achieve a prolonged drug release, suggesting that the diffusion rate of CFT from the NP can me modified by increasing the viscosity of solution.

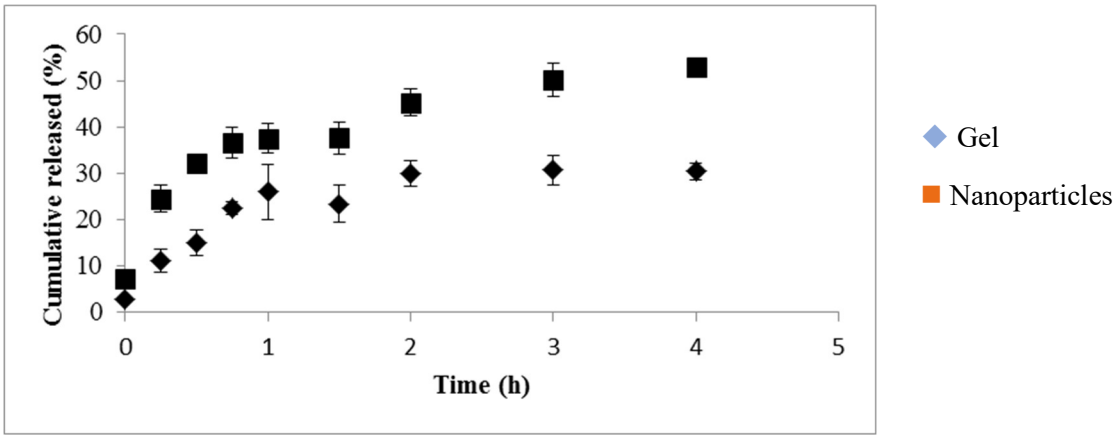


Figure 3.4 - Release profiles of CFT from CS/HA/TPP nanoparticles in 10 mM PBS pH 7.4 at 37 ºC (mean ± SD, n = 3).

CFT release values were fit to different mathematical models (zero-order, first-order, Higuchi and Kormeyer-Peppas). The release data for NP and NP+HPMC best fitted to the Korsmeyer-Peppas model (table 3.9) This model of drug release is used when more than one type of mechanism of drug release is involved. Moreover, the Korsmeyer-Peppas release model exponent, *n*, is less than 0.5 for both formulations which suggests that the Fickian diffusion is the controlling factor in drug release. (Dash *et al*, 2010) The release rate of CFT through the vehicle network to the external medium is significantly dependent on the rate of molecular diffusion of CS/TPP/HA nanoparticulate system.

Table 3.9- Mathematical models and respective parameters (correlation coefficients and kinetic constants) obtained from fitting the CFT release experimental data.

	Zero-order		**First-order**		**Higuchi**		**Korsmeyer–Peppas**		
	r^2	k_0 (µg/h)	r^2	k_1 (h^{-1})	r^2	k_H ($h^{-0.5}$)	r^2	k_{KP} (h^n)	n
NP	0.87	6.22	0,89	0.14	0,87	12,44	0,98	0,60	0.27
NP Gel	0.86	6.13	0,89	0.11	0,86	12,25	0,91	0,59	0.28

r^2: correlation coefficient; k_0: zero-order release constant; k_1: first-order release constant; k_H: Higuchi constant k_{KP}: Korsmeyer–Peppas constant; n: release mechanism exponent.

3.7 *In vitro* ceftazidime permeation studies

Permeation studies were developed for NP in solution and incorporated in HPMC using Franz cells with cellulose membranes.

There is a very similar profile between the permeation of CFT through the membranes when administered in NP solution and NP+HPMC. The amount of CFT released from NP in solution is slightly higher than from NP in HPMC. In this case the maximum release of CFT is 20% .

CFT permeation from NP in HPMC is smaller than NP in solution and it is expected because gel formulation has a higher viscosity resulting in a more compact polymer matrix that reduces the degradation of the polymer and/or the diffusion of the loaded CFT from the NP. As observed before in the release studies, the permeation profiles also show an initial burst phase due to the absorbed CFT in the surface of NP that is followed by a slow permeation of the trapped drug (figure 3.5).

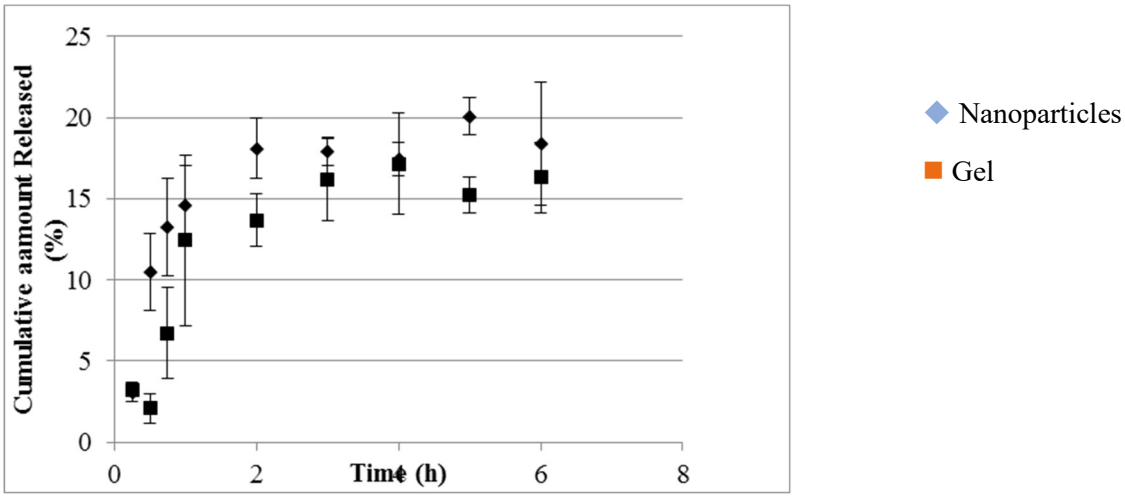


Figure 3.5 – Permeation profile of NP solution and NP gel in 10mM PBS pH 7.4 through a cellulose membrane at 37ºC (mean ± SD, n=6)

3.8 Mucoadhesive studies

The interaction and stability of CS/TPP/HA NP in solution and incorporated in HPMC gel in the presence of mucin was determined by measuring the viscosity of a mucin dispersion before and after incubating with the NP. This determination is essential because the blink process requires a low tear viscosity to avoid damaging the corneal epithelium so it is ideal that the viscosity of the NP solution does not increase significantly when interacting with mucins. Mucins are elongated molecules composed of a chain containing an amide group that confer its hydrophobic characteristics and branching chains of sugar enriched with serine, threonine and proline, with hydroxyl, carboxylic and sulfate groups that result in highly hydrophilic nature. Furthermore, mucin contains at the end of its molecule an amino terminus and a carboxyl terminus (Sheardown and Lorentz, 2014).

It was studied the effect of interaction of NP in solution and in HPMC with mucin in the viscosity of the system, because a higher interaction with mucin is related with higher viscosity.

The results of the study with an Ostwald viscometer presented in figure 3.6. show an increase of viscosity for CFT NP when compared with empty NP. This difference is more evident in the presence of HPMC allowing to understand that CFT plays an important role in the increasing of viscosity.

The developed system with CFT NP and HPMC, in the presence of mucin, shows an increase of viscosity, allowing to concluded that mucoadhesion is ensured. This interaction between NP and mucin is due to the fact that the carboxylic acid groups of mucin are ionized at the pH value of 6 and therefore they are freely accessible for interaction with the positively charged amine groups of CS (present in the NP).

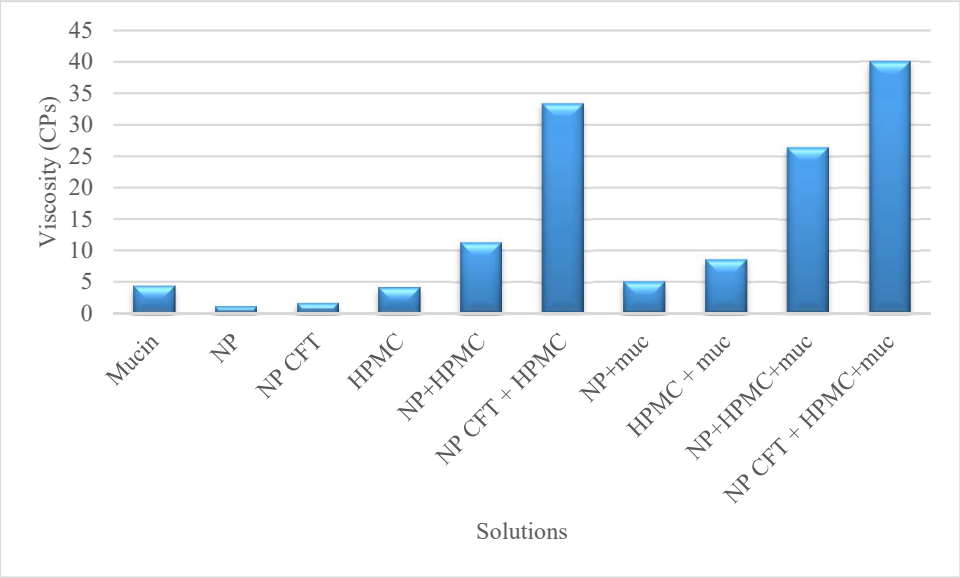


Figure 3.6 - Viscosity determination for NP and HPMC in presence and absence of mucin 2% (w/V)

ZP results of the nanoparticles incubated with mucin dispersion suggest that interactions between mucin and NP are present. The ZP profiles obtained for tested solutions with mucin and with water support this observation; the ZP values are reduced for the solutions containing mucin. These results are consistent with the ones obtained by De Campos *et al* (2004) in experiments with CS and mucin. This reduction can be attributed to the ionic interactions between the negatively charged mucin particles and the positive charges of CS/TPP/HA NP, resulting these interactions in a reduced surface charge and ZP value (table 3.10).

Table 3.10 - Determination of zeta potential for NP and NP in HPMC for different concentration of mucin (mean ± SD, n=3)

Mucin Conc.	Mucin	NP	Gel+NP
(mg/mL)	ZP (mv)	ZP (mv)	ZP (mv)
0		+34±1	+2±0
5	-24±1	-5±1	-5±1
10	-21±1	-15±1	-8±0
20	-18±0	-14±1	-12±2

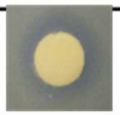
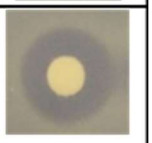
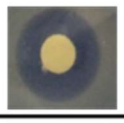
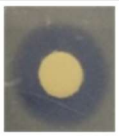
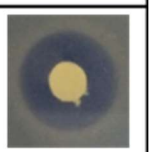
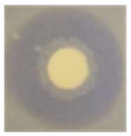
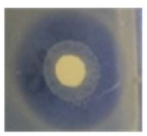
3.9 Microbiological assay

3.9.1 Agar diffusion method

The agar diffusion method intended to test the susceptibility of *P. aeruginosa* to CFT. It was used free CFT in solution, encapsulated CFT in NP and encapsulated CFT in NP within HPMC gel. In this method it is the diameter of the inhibition zone that allows to verify the efficacy of the antibiotic against the pathogen, because the diameter of the inhibition zones are proportional to the sensitivity of the microorganism (Bonev et al, 2008).

As expected, and as a control, there is no inhibition zone (0mm) for empty NP (table 3.11). The obtained results suggest a difference in the antimicrobial activity of CFT in the NP formulation and in NP+HPMC gel formulation. The inhibition zone of NP+HPMC gel formulation was smaller than the ones we obtained for the NP solutions. It can be explained as the fact that HPMC gel acts as a barrier for drug diffusion out of the polymeric matrix and this results in a decreased concentration of antibiotic near the bacterial cells and lowest antimicrobial effects. The solution of free CFT obtained the higher inhibition zone however this value is not very distant from those resulting from the NP formulations.

Table 3.11 – Antimicrobial activity of NP solutions, NP gel and free CFT against bacterial test organism (P. aeruginosa) - inhibition zones in mm

Solution	Volume (µL)	Inibition zone (φ = mm)	
Empty NP	20	0	
CFT NP	5	1.3	
	10	13.1	
	20	15.7	
Empty NP + HPMC	20	0	
CFT NP + HPMC	5	1.3	
	10	11.4	
	20	12.8	
Free CFT	5	13.7	
	10	16.5	
	20	19.2	

3.9.2- Microtitre plate antibacterial assay

This microtitre plate antibacterial assay was adapted from the method previously described by Sarker *et al*. (2007) and according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) by broth microdilution method. To evaluate the possible differences in the antimicrobial effect of CS/TPP/HA NP prepared in different times, NPs with 1 day and 1 month were teste. In addition, the antimicrobial effect of NP in gel and of free CFT was tested. The final concentration of free CFT and CFT in NP used in this assay was 100µg/mL. The results are shown on the table 3.12.

Table 3.12 – Antimicrobial activity of NP solutions, NP gel and free CFT against bacterial test organism (*P. aeruginosa*). MIC results in µg/ml.

Solution	**MIC (µg/mL)**
NP solution	0.78
NP solution 1 month 4ºC	1.56
NP Gel solution	1.56
NP Gel solution 1 month 4ºC	3.13
Free CFT	0.78

The results of the microtitre plate antibacterial assay (table 3.12) are in accordance with the agar diffusion test. The MIC value of the NP formulation and free CFT solution was found to be 0.78µg/mL for the tested *P. aeruginosa*. Values in conformity with the work of Sueke *et al* 2010. The same inhibitory concentration for these formulations reveal that the incorporation of CFT into the matrix of CS/TPP/HA NPs was successful in maintaining CFT antimicrobial activity. The MIC value of the NP gel formulation was significantly higher (1.56µg/mL) probably for the same reason described in the section above. It is noted an increase of the MIC after one month of storage at 4ºC for both NP and NP Gel formulations. The increase of the MIC can be related to the degradation of the non-encapsulated antibiotic.

Previous investigations have proposed different mechanisms of antibacterial activity of NP. Some studies refer that NP can fuse with the microbial cell wall and then the antibiotic is released within the cell wall and membrane (Zhang *et al*, 2010). Others report that NP are able to bind to the cell wall and act as a drug depot releasing the antibiotic

continuously. The released drug diffuses into the inner compartments of the bacteria (Zhang, 2010). To determine the exact mechanism *in vivo* of CS/TPP/HA NP more tests will be necessary.

3.10 *In vitro* cell assays

3.10.1 Cell viability

The results of cell viability for the different tested samples obtained from the Alamar Blue and PI assays are presented in Figure 3.7. These results shown a good profile in terms of cell viability of ARPE-19 and HEK 293T cell lines, measured by a metabolic assay and membrane integrity assay. The results showed that these formulations were not toxic for the cells at concentrations below 200 μg/mL. These results are in agreement with previously published data where the tested formulation of NP showed 100% of cell viability (de la Fuente et al, 2010).

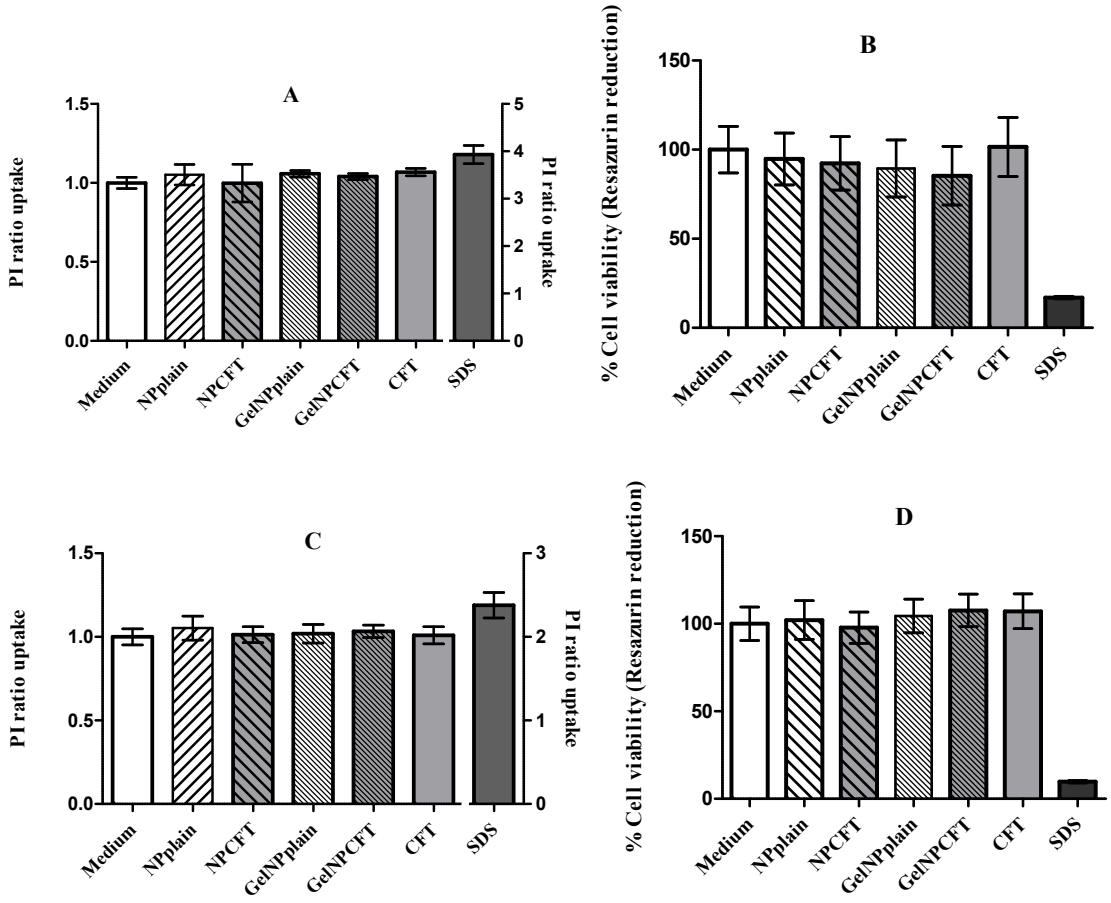


Figure 3.7- Cell viability of HEK293T (A;B) and ARPE-19 (C,D) cell lines exposed for 24h to 200 µg/mL of formulations (A,C- PI ratio uptake; B,D- Rezasurin reduction) (mean ± SD, n=6)

3.10.2 Oxidative stress assay

The ability of NPs to induce intracellular oxidant production in HEK 293T and ARPE-19 cells was assessed using DCF fluorescence, which reacts with oxygen radicals. All samples do not significantly increase the intracellular ROS production after 24h of exposition for the two cell lines tested as shown in Figure 3.8.

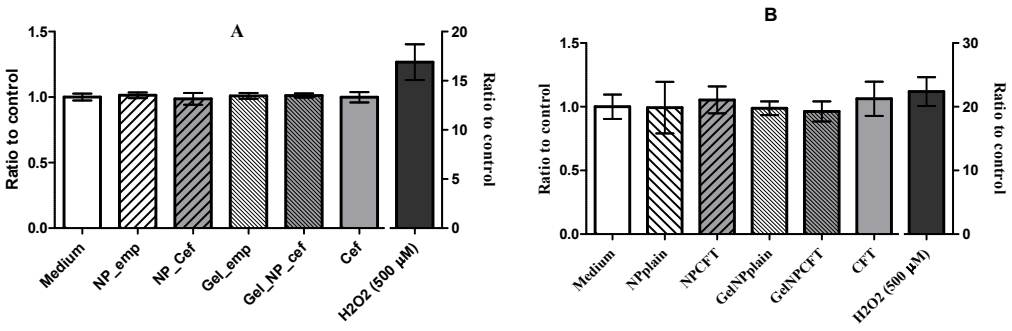


Figure 3.8- Reactive oxygen species (ROS) (Cells exposed 24h to 200µg/mL of formulations; A- HEK293T cell line and B-ARPE-19 cell line) (mean ± SD, n=6)

4. Conclusion

The main purpose of this research work was the development of an effective CFT nanocarrier system that could be used to deliver the antibiotic directly into the eye, in order to act as a future therapy against *Bacterial Keratitis*.

CFT has been proven to be a powerful antimicrobial agent against a wide range of microorganisms responsible for eye infections, like *Staphylococcus aureus and Pseudomonas aeruginosa.*

CFT- loaded CS/HA formulations were characterized for their physiochemical properties revealing distribution sizes between 631.6±11.25 and 742.9±6.58 nm, and zeta potential values between 37.2±0.7 and 38.8±0.08 mV, being suitable for ocular applications. CFT encapsulation efficiency of CFT was 78.4±0.96% into NP showing a low waste of the antibiotic. Positive charge of CS, allows the interaction of NP with hydroxyl and amino groups with the sialic groups of mucin in the mucus layer, prolonging the residence time in the eye.

CFT NPs have as a vehicle, HPMC 0.5% HV and this cellulose derivative presented the desired characteristics: adequate viscosity that allows prolonged contact time of the drug in the ocular globe and does not cause blurred vision; the pH value around 7.4 and the osmolality near 300mOsm/kg are similar to the physiological fluids and considered adequate.

The mucoadhesivity studies for the formulations (NPs included in HPMC) allows to confirm the increased interaction with the ocular mucin and increased residence time of the antibiotic in the eye.

The results of antimicrobial assays reveal that encapsulating CFT in NP does not change its antimicrobial activity which remains sufficient to inhibit bacterial growth. Also, *in vitro* cytotoxicity studies revealed that formulations were not toxic for ARPE-19 and HEK 293T cells lines.

To sum up, developed chitosan coated hyaluronic acid ceftazidime loaded nanoparticles seem to be an interesting and potential therapy for bacterial keratitis treatment.

This work constitutes an important “proof of concept” step and can be completed in the future by studying other biological and pharmaceutical properties of the developed nanoparticles, including: sustained release testing, evaluation of nanoparticles stability when in contact with ocular fluids, namely tear film, and retain the long-term stability of developed nanoparticles after freeze-drying and sterilization.

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